



# Long-term impact of sewage sludge application on soil microbial biomass: An evaluation using meta-analysis<sup>☆</sup>



Alex Charlton<sup>a</sup>, Ruben Sakrabani<sup>a,\*</sup>, Sean Tyrrel<sup>a</sup>, Monica Rivas Casado<sup>a</sup>,  
Steve P. McGrath<sup>b</sup>, Bill Crooks<sup>c</sup>, Pat Cooper<sup>d</sup>, Colin D. Campbell<sup>d,e</sup>

<sup>a</sup> School of Water, Energy and Environment, Cranfield University, UK

<sup>b</sup> Rothamsted Research, Harpenden, Herts AL5 2JQ, UK

<sup>c</sup> SRUC, West Mains Road, Edinburgh, Scotland EH9 3JG, UK

<sup>d</sup> James Hutton Institute, Craigiebuckler, Aberdeen, Scotland, AB15 8QH, UK

<sup>e</sup> Department of Soil and Environment, Swedish Agricultural Sciences University, Uppsala, Sweden

## ARTICLE INFO

### Article history:

Received 12 May 2016

Received in revised form

21 July 2016

Accepted 22 July 2016

Available online 4 August 2016

### Keywords:

Sewage sludge

Microbial biomass carbon

Heavy metals

Meta-analysis

## ABSTRACT

The Long-Term Sludge Experiments (LTSE) began in 1994 as part of continuing research into the effects of sludge-borne heavy metals on soil fertility. The long-term effects of Zn, Cu, and Cd on soil microbial biomass carbon ( $C_{mic}$ ) were monitored for 8 years (1997–2005) in sludge amended soils at nine UK field sites. To assess the statutory limits set by the UK Sludge (Use in Agriculture) Regulations the experimental data has been reviewed using the statistical methods of meta-analysis. Previous LTSE studies have focused predominantly on statistical significance rather than effect size, whereas meta-analysis focuses on the magnitude and direction of an effect, i.e. the practical significance, rather than its statistical significance. The results presented here show that significant decreases in  $C_{mic}$  have occurred in soils where the total concentrations of Zn and Cu fall below the current UK statutory limits. For soils receiving sewage sludge predominantly contaminated with Zn, decreases of approximately 7–11% were observed at concentrations below the UK statutory limit. The effect of Zn appeared to increase over time, with increasingly greater decreases in  $C_{mic}$  observed over a period of 8 years. This may be due to an interactive effect between Zn and confounding Cu contamination which has augmented the bioavailability of these metals over time. Similar decreases (7–12%) in  $C_{mic}$  were observed in soils receiving sewage sludge predominantly contaminated with Cu; however,  $C_{mic}$  appeared to show signs of recovery after a period of 6 years. Application of sewage sludge predominantly contaminated with Cd appeared to have no effect on  $C_{mic}$  at concentrations below the current UK statutory limit.

© 2016 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

Over the past two decades the recycling of sewage sludge has remained a significant management problem, with the total quantities of sewage sludge produced annually in the UK increasing to an estimated 1.6 million tonnes (dry solids) in 2010 (Defra, 2007b). Application of sewage sludge to agricultural land is currently seen as the best practical environmental option for recycling this material within the UK (Defra, 2007b; Gendebien et al., 1999; Gendebien et al., 2010; Water UK, 2010). However,

due to the sources of wastewater, both domestic and industrial, and the nature of wastewater treatment processes themselves, sewage sludge frequently contains concentrations of potentially toxic heavy metals that are significantly greater than the background concentrations found in soils (Berrow and Webber, 1972; Smith, 1996; Thornton et al., 2001). This is problematic as heavy metals are extremely persistent and can contaminate soils for decades (Alloway and Jackson, 1991; McGrath, 1987). Although the quality of sludge materials has improved in recent years, due to declining metal content (CEC, 2010; Gendebien et al., 1999; Smith, 1996; Thornton et al., 2001), there remains an understandable concern that increasing the quantities of sewage sludge used in agriculture could potentially lead to an accumulation of heavy metals within the environment and a long-term reduction in the quality of agricultural soils.

<sup>☆</sup> This paper has been recommended for acceptance by B. Nowack.

\* Corresponding author.

E-mail address: [r.sakrabani@cranfield.ac.uk](mailto:r.sakrabani@cranfield.ac.uk) (R. Sakrabani).

In order to prevent a potentially hazardous accumulation of heavy metals the UK Sludge (Use In Agriculture) Regulations (UK-SR) set statutory maximum limits for the total concentrations of cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn) permitted in sludge amended soils, as these are considered to pose the greatest risk to soil and human health (SI, 1989). The UK-SR are further supported by an industry 'Code of Practice', drafted by the UK Department of Environment (DoE, 1996), which provides advisory limits for an additional five heavy metals (Table 1). Following implementation of the UK-SR, two independent scientific reviews were conducted to determine possible risks to food safety, assess the potential long-term impacts of repeated sludge application to agricultural land, and confirm that the legislation put in place was sufficient to protect soil quality. These were carried out by the Steering Group on Chemical Aspects of Food Surveillance (MAFF/DoE, 1993a) and an independent scientific committee (MAFF/DoE, 1993b); both commissioned on behalf of the UK Ministry for Agriculture, Fisheries and Food and the UK Department of Environment (MAFF/DoE, now combined as the Department for Environment, Food and Rural Affairs (Defra)). Overall it was concluded that heavy metal uptake by plants was unlikely to pose a significant risk to food safety (MAFF/DoE, 1993a); hence the limits proposed by the UK-SR were deemed sufficient to protect plants, animals, and humans from metal toxicity. However this could not be said for soil microorganisms (MAFF/DoE, 1993b).

Evidence for the impact of heavy metals on the soil microbial community was only beginning to emerge when the UK-SR were first drafted; therefore soil microorganisms were not considered when establishing the statutory limits. However there was concern that a decrease in the diversity and activity of soil microorganisms, due to heavy metal toxicity, could disrupt biogeochemical processes essential to crop production (MAFF/DoE, 1993b). The definition of soil as a 'living system' emphasises the role of microorganisms in the mineralisation of soil organic matter and the cycling of soil nutrients (Doran and Safley, 1997) for which the soil microbial community has been described as 'the eye of the needle through which all organic materials must pass' (Jenkinson, 1977). The long-term impact of heavy metal contamination, on microbial communities present within sludge amended soils, is still not fully understood (Giller et al., 1998, 1999, 2009) and a range of biological indicators, such as microbial biomass carbon (Jenkinson and Ladd, 1981), specific respiration rate (Anderson and Domsch, 1993), and soil enzyme activities (Dick, 1994) have been suggested as a means

of gauging the extent to which soil microbial communities are under environmental stress (Brookes, 1995; Ritz et al., 2009; Schlöter et al., 2003). A number of advanced molecular techniques, such as the analysis of phospholipid fatty acid biomarkers (Zelles, 1999) and multiplex-terminal restriction length fragment polymorphism (Macdonald et al., 2011), are also becoming increasingly available to environmental scientists allowing more detailed investigation of soil microbial communities and the mechanisms by which heavy metal contamination can disrupt essential soil processes (Ritz et al., 2009). Of these indicators, soil microbial biomass carbon ( $C_{mic}$ ), taken as a gross measurement of the microbial community size, has frequently been used to investigate the long-term impact of sludge-borne heavy metals on microorganisms within the soil environment (Abaye et al., 2005; Brookes and McGrath, 1984; Chander and Brookes, 1991, 1993; Fließbach et al., 1994). The microbial biomass of soil comprises the total mass of fungi, bacteria, protozoa, and algae, per unit weight of soil and is regarded as an undifferentiated single compartment for the purpose of studying energy flows and mineral fluxes within the soil environment. However due to limitations in experimental methods, as well as the natural spatial and temporal variation in microbial growth and activity in soils, results are often difficult to compare (Brooks et al., 2007; Martens, 1995).

One of the major obstacles encountered when setting regulatory limits remains a lack of data available to establish dose-response curves, giving more accurate estimates of the minimum heavy metal concentrations having an adverse effect on soil microorganisms. The need for long-term monitoring of soil microbial communities in contaminated soils is recognised by several authors (McBride, 2003; McGrath et al., 1994, 1995). Therefore, following the review of the UK-SR, a series of 'Long-Term Sludge Experiments' (LTSE) were established by Defra as part of a continuing investigation into the effects of heavy metals on soil fertility and the long-term impact of sewage sludge applications on soil microorganisms (MAFF/DoE, 1993b). The aim of this paper is to provide an additional review of the LTSE, using the statistical methods of meta-analysis to give a clear overview of the experimental data. Meta-analysis is becoming increasingly applied within the environmental sciences to help understand a wide range of research questions. Application of meta-analysis to the LTSE data set will allow for an evaluation of previous conclusions and an assessment of the statutory limits set by the UK-SR by determining the overall long-term impact of heavy metal contamination on  $C_{mic}$  in sludge amended soils.

**Table 1**  
Maximum concentrations of heavy metals in sludge amended soils, and average annual rate of application over a 10 year period, permitted by the UK Sludge (Use In Agriculture) Regulations (SI, 1989).

	Soil limit value (mg kg <sup>-1</sup> )				Maximum permissible annual average over 10 years (kg ha <sup>-1</sup> y <sup>-1</sup> )
	pH 5.0 < 5.5	pH 5.5 < 6.0	pH 6.0–7.0	pH > 7.0	
Zinc	200	250 (200) <sup>a</sup>	300 (200)	450 (300)	15
Copper	80	100	135	200	7.5
Nickel	50	60	75	110	3
For pH 5.0 and Above					
Cadmium	3				0.15
Lead	300				15
Mercury	1				0.1
Chromium <sup>b</sup>	400				15
Molybdenum <sup>b</sup>	4				0.2
Selenium <sup>b</sup>	3				0.15
Arsenic <sup>b</sup>	50				0.7
Fluoride <sup>b</sup>	500				20

<sup>a</sup> Values in parenthesis are UK advisory limits (MAFF/DoE, 1993b).

<sup>b</sup> Values are advisory limits from UK Department of Environment 'Code of Practice' (DoE, 1996).

## 2. Methods

### 2.1. Long-term sludge experiments

The 'Long-Term Sludge Experiments' (LTSE), conducted by ADAS, Rothamsted Research, and WRc in England and Wales, and the Macaulay Land Use Research Institute (now the James Hutton Institute) and SAC (now SRUC) in Scotland, began in 1994 at nine UK field sites (Fig. 1), chosen to provide a range of soil types (Table 2) from varying climatic regions (Defra, 2002, 2007a; Gibbs et al., 2006). Soil pH was kept constant at all sites for the duration of the experiment by application of lime (with the exception of the calcareous site, Shirburn, which was left at the natural pH of 8). The sites in England and Wales were maintained at a pH of 6.5, whereas soil pH at the Scottish sites, Auchincruive and Hartwood, were kept at 6.0 and 5.8, respectively. An Italian ryegrass was sown at each of the field sites during the autumn of 1997, following the final applications of sewage sludge, and subsequently harvested in summer the following year (Defra, 2002). Three of the field sites (Auchincruive, Hartwood, and Pwllpeiran) continued to be managed as grassland for the duration of the LTSE, whereas a ley/arable cropping regime was implemented at the remaining sites (Table 3). Spring wheat (Chablis) was sown in February/March during 1999, 2001, 2003, and 2005, and harvested during the summer each year; each site was then sown with Italian ryegrass in the autumn to be harvested during summer in the intermediate years (Defra, 2002, 2007a).

Five sludge cake treatments were applied annually to experimental plots over the course of four years (1994–1997); in each case sludge applications were made during summer (Defra, 2002). Three of the sludge treatments contained elevated concentrations of either Zn, Cu, or Cd, whereas the remaining 'uncontaminated'

treatments contained concentrations of heavy metals typical for sludge produced in the UK at that time (Control 1 and Control 2; Table 4). The same sludge cake materials were transported to each site to ensure that exactly the same treatments were applied using the same methods of application. Sludge treatments were replicated three times ( $n = 3$ ) in fully randomised blocks consisting of  $6 \text{ m} \times 8 \text{ m}$  plots and annually cultivated by spading machine to a depth of 25 cm (Gibbs et al., 2006); field sites were cultivated a further three times during 1998 (Defra, 2002). Contaminated sludge treatments were applied in increasing quantities to gradually elevate the total metal concentration in the receiving soils and establish dose-response curves for the three heavy metals (Table 5). Application of the Zn, Cu, and Cd sludge treatments were then supplemented with corresponding sludge material (i.e. anaerobically digested sludge for Zn and Cd, and undigested raw sludge for Cu (Table 4)) from Controls 1 and 2 to ensure uniform quantities of organic carbon were applied across all levels of the dose-response curves (Gibbs et al., 2006). Applications of the uncontaminated controls were made to separate plots, at the same overall rate, to control for the effect of applying organic carbon to soil (Table 2); an untreated soil was also included in the experimental design. Note that the rate of organic carbon application for digested and undigested sludge treatments varied between sites (Table 2). Each dose-response curve was comprised of four levels of increasing metal concentration, ranging from  $150$  to  $450 \text{ mg kg}^{-1}$ ,  $50$ – $200 \text{ mg kg}^{-1}$ , and  $1$ – $4 \text{ mg kg}^{-1}$  for Zn, Cu, and Cd, respectively (Table 5); an additional level was included for the Shirburn site due to the calcareous nature of the soil. However, in some cases, particularly for soils receiving the Zn sludge treatment, the target metal concentrations were not achieved at all of the sites (Table 6). This is likely due to difficulties encountered during cultivation when incorporating the sludge cake material into the top soil; hence in some cases a homogeneous distribution of the sludge treatment was not achieved (Gibbs et al., 2006; Defra, 2008).

Following the final applications of sewage in 1997, approximately 55 and 36% of the applied organic carbon remained in soils receiving the digested and undigested sludge treatments, respectively (Table 2). In each case  $C_{mic}$  had increased in comparison to untreated soil at all nine of the LTSE field sites (Gibbs et al., 2006). Significant ( $p < 0.05$ ) decreases in  $C_{mic}$  were observed at the Rosemaund and Gleadthorpe field sites where  $C_{mic}$  in soils receiving the Zn and Cu sludge treatments, at dose-response level 3, was, respectively, 20–30%, and 45%, lower in comparison to soils receiving the corresponding uncontaminated sludge treatments. Soil microbial biomass was subsequently monitored for eight years, with sampling events occurring in 1999, 2001, 2003, and 2005. No significant differences were reported between soils receiving contaminated and uncontaminated sludge treatments during 1999, although regression analysis did show a negative relationship between  $C_{mic}$  and the total concentrations Zn and Cu at a several of the LTSE field sites. By 2001, the majority of significant decreases reported were observed at the Rosemaund field site, whereas for 2003 and 2005, the majority of significant differences were seen at the Woburn and Gleadthorpe field sites, respectively; with the most significant differences overall observed at Gleadthorpe (Defra, 2002, 2007a). A summary of statistically significant results is presented as supplementary information (Appendix 1).

### 2.2. Data sources and treatment

A common tendency of primary studies is to focus predominantly on statistical significance rather than effect size. It is often assumed that the absence of statistical significance provides evidence for the null hypothesis, hence the total number of significant results is compared to that of non-significant results to determine

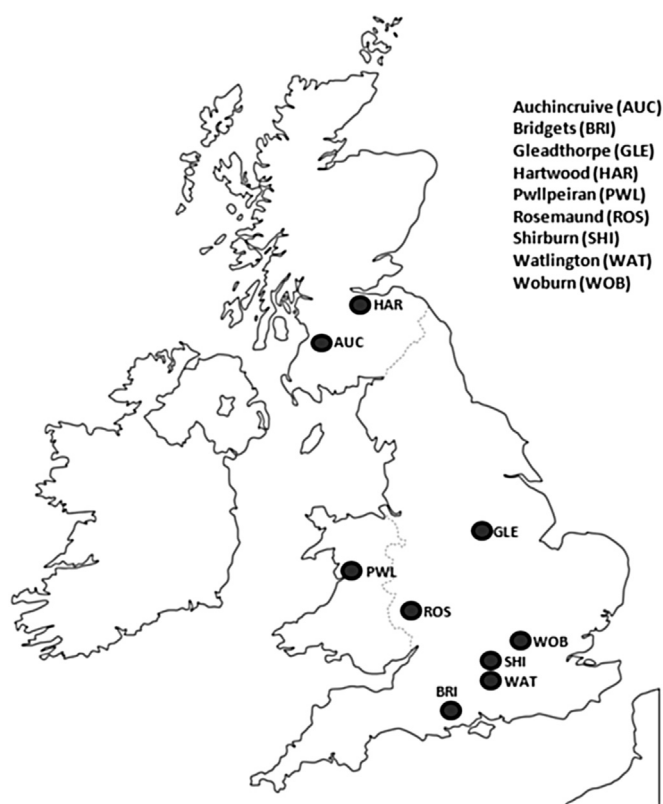


Fig. 1. Location of the 'Long-Term Sludge Experiment' field sites (Adapted from Gibbs et al., 2006).

**Table 2**

Soil properties at each of the 'Long-Term Sludge Experiment' field sites in 1994, prior to the application of sludge treatments (Adapted from Gibbs et al., 2006).

	AUC <sup>a</sup>	BRI	GLE	HAR	PWL	ROS	SHI	WAT	WOB
Sand (%)	51	10	71	59	24	8	44	56	80
Silt (%)	29	60	22	20	53	67	36	28	12
Clay (%)	20	30	7	21	23	25	20	16	8
Texture	Sandy Clay Loam	Silt Clay Loam	Sandy Loam	Sand Clay Loam	Clay Loam	Silt Clay Loam	Clay Loam (Calcareous)	Sandy Loam	Loamy Sand
pH	6.0	6.8	7.1	5.8	5.4	7.0	8.0	7.4	7.2
Organic C (%)	2.5	1.5	1.2	4.7	3.3	1.7	3.0	1.3	1.3
Fe <sub>2</sub> O <sub>3</sub> (%)	4.24	4.00	1.66	3.32	5.69	4.77	3.25	3.71	2.90
Al <sub>2</sub> O <sub>3</sub> (%)	3.54	5.10	1.51	7.87	4.97	5.77	2.45	2.25	1.13
MnO <sub>2</sub> (%)	0.12	0.23	0.06	0.96	0.15	0.16	0.12	0.09	0.03
Total Zn (mg kg <sup>-1</sup> )	82.4 (0.42) <sup>b</sup>	49.4 (0.61)	34.4 (0.92)	72.3 (1.87)	140 (4.59)	77.8 (0.98)	68.5 (0.94)	43.3 (0.49)	44.8 (1.06)
Total Cu (mg kg <sup>-1</sup> )	22.6 (0.14)	12.2 (0.24)	7.4 (0.28)	19.8 (0.33)	13.2 (0.74)	17.3 (0.09)	13.1 (0.12)	11.4 (0.11)	13.9 (0.36)
Total Cd (mg kg <sup>-1</sup> )	0.33 (<0.01)	0.95 (0.04)	0.17 (0.01)	0.23 (0.01)	0.15 (<0.01)	0.24 (0.01)	0.37 (0.01)	0.27 (0.01)	0.12 (<0.01)
Biomass C (mg kg <sup>-1</sup> )	428 (6.8)	293 (11.2)	66 (3.5)	663 (24.8)	609 (23.8)	419 (10.0)	1094 (29.5)	278 (10.0)	108 (4.5)
Digested sludge (t C ha <sup>-1</sup> yr <sup>-1</sup> )	17.25	12.00	17.25	18.50	8.25	14.25	9.25	15.75	20.25
Undigested SLUDGE (t C ha <sup>-1</sup> yr <sup>-1</sup> )	20.75	12.25	17.00	20.25	7.50	14.25	10.25	16.75	20.25

<sup>a</sup> For abbreviations see Fig. 1.<sup>b</sup> Values in parenthesis are standard error (n = 3).**Table 3**

Cropping regimes implemented at each of the Long-Term Sludge Experiment field sites. Italian rye grass was sown in autumn and harvested during summer the following year as indicated. Chablis wheat was sown in spring and harvested the same year during summer as indicated. Soil samples were collected from each of the field sites during March/April in 1999, 2001, 2003, and 2005 (Adapted from Defra, 2002, 2007a).

Field site	Cropping regime							
	1998	1999	2000	2001	2002	2003	2004	2005
Auchincruive	Grass	Grass	Grass	Grass	Grass	Grass	Grass	Grass
Hartwood	Grass	Grass	Grass	Grass	Grass	Grass	Grass	Grass
Pwllpeiran	Grass	Grass	Grass	Grass	Grass	Grass	Grass	Grass
Bridgts	Grass	Wheat	Grass	Wheat	Grass	Wheat	Grass	Wheat
Gleadthorpe	Grass	Wheat	Grass	Wheat	Grass	Wheat	Grass	Wheat
Rosemaund	Grass	Wheat	Grass	Wheat	Grass	Wheat	Grass	Wheat
Shirburn	Grass	Wheat	Grass	Wheat	Grass	Wheat	Grass	Wheat
Watlington	Grass	Wheat	Grass	Wheat	Grass	Wheat	Grass	Wheat
Woburn	Grass	Wheat	Grass	Wheat	Grass	Wheat	Grass	Wheat

whether an effect exists or not (Hedges and Olkin, 1980). In the case of the LTSE, from a total of 471 measurements of  $C_{mic}$ , over the course of 8 years, only 32 were reported to be significantly lower in soils treated with contaminated sludge in comparison to soils receiving the corresponding uncontaminated controls (Appendix 1). Similarly, of a possible 108 linear regressions between  $C_{mic}$  and the total concentrations of Zn, Cu, and Cd, only 33 were reported to be statistically significant (Appendix 1). However, non-significant results can simply be due to low statistical power where small sample sizes have been used in primary studies (Borenstein, 2000). In this case the contaminated and uncontaminated sludge treatments applied at the LTSE were applied in

**Table 5**

Target metal concentrations for the dose-response curves established at each of the Long-Term Sludge Experiment field sites (Adapted from Gibbs et al., 2006).

Dose-response curve	Target metal concentration in soil (mg kg <sup>-1</sup> )		
	Zinc	Copper	Cadmium
Level 1	150	50	1
Level 2	250	100	2
Level 3	350	150	3
Level 4	450	200	4
Level 5 <sup>a</sup>	600	275	5

<sup>a</sup> Additional level only applied at the Shirburn (SHI) site to account for potential calcareous soil-metal interactions.

triplicate (n = 3), which may not be sufficient to overcome the natural variation in  $C_{mic}$  observed within the soil environment. Therefore it could be that the effect of applying contaminated sewage sludge caused drastic decreases in  $C_{mic}$  at some of the field sites, or was consistently negative across all field sites, but produced non-significant results due to a low sample size; hence the overall effect may have been overlooked. Meta-analysis approaches this ambiguity by focussing on the magnitude ( $|x|$ ) and direction ( $\pm$ ) of an effect, i.e. the practical significance, rather than its statistical significance (Borenstein, 2000; Borenstein et al., 2009). The goal of meta-analysis is also to test the null hypothesis of no effect, however, this is achieved by establishing a common framework in which direct comparisons can be made between independent primary studies ( $i$ ). The observed effects ( $R_i$ ) are then combined to give an overall summary effect ( $M$ ) across a number of primary studies ( $k$ ), thus giving a more precise estimation of effect size. Combining studies in this way markedly increases statistical power, by

**Table 4**

Properties of sludge treatments applied over the course of four years (1994–1997) at each of the Long-Term Sludge Experiment field sites (Adapted from Gibbs et al., 2006).

	Control 1 (digested sludge)	Control 2 (undigested sludge)	Zinc (digested sludge)	Copper (undigested sludge)	Cadmium (digested sludge)
Dry matter (%)	18.3	36.7	23.5	18.0	67.8
Organic C (%)	38.1	42.9	31.6	37.6	12.9
pH	7.3	7.3	7.5	5.2	6.8
Zn (mg kg <sup>-1</sup> )	560	490	<b>6000<sup>a</sup></b>	550	1100
Cu (mg kg <sup>-1</sup> )	590	450	<b>1400</b>	<b>5050</b>	540
Cd (mg kg <sup>-1</sup> )	1.8	1.7	11.2	0.7	<b>44</b>

<sup>a</sup> Values in bold are above EU Sludge Directive lower limits for the respective metal concentrations in sludge: Zn, 2500–4000 mg kg<sup>-1</sup>; Cu, 1000–1750 mg kg<sup>-1</sup>; Cd, 20–40 mg kg<sup>-1</sup> (CEC, 1986).



**Table 6**

Total metal concentrations measured for each of the 'Long-Term Sludge Experiment' field sites after the final applications of contaminated sludge in 1997 (Adapted from Gibbs et al., 2006).

	Metal concentrations measured in 1997 (mg kg <sup>-1</sup> )								
	AUC <sup>a</sup>	BRI	GLE	HAR	PWL	ROS	SHI	WAT	WOB
Zn 1	147 (1.8) <sup>b</sup>	202 (27.7)	134 (6.6)	171 (16.8)	212 (8.3)	152 (5.3)	185 (5.9)	203 (14.0)	125 (14.1)
Zn 2	236 (10.2)	299 (17.9)	232 (49.0)	240 (11.6)	274 (2.4)	251 (4.5)	298 (20.4)	304 (35.5)	211 (8.1)
Zn 3	314 (8.1)	414 (9.3)	334 (44.4)	406 (30.9)	371 (17.1)	444 (8.0)	345 (5.4)	459 (21.6)	304 (24.9)
Zn 4 <sup>c</sup>	342 (30.3)	459 (51.0)	291 (29.4)	443 (4.2)	370 (20.5)	421 (34.7)	414 (17.5)	539 (10.6)	224 (16.4)
Cu 1	66 (4.2)	75 (2.7)	69 (6.8)	74 (8.4)	81 (2.9)	74 (5.5)	76 (7.2)	109 (0.3)	56 (3.3)
Cu 2	103 (9.1)	136 (2.4)	120 (18.3)	129 (6.6)	182 (13.7)	137 (17.7)	140 (5.9)	178 (28.1)	92 (10.3)
Cu 3	140 (17.0)	212 (1.0)	188 (39.4)	195 (15.7)	202 (15.3)	202 (38.7)	171 (2.1)	254 (14.1)	161 (15.0)
Cu 4 <sup>c</sup>	206 (31.6)	209 (3.8)	166 (23.6)	239 (17.1)	234 (37.9)	219 (21.8)	224 (12.6)	309 (26.4)	188 (37.6)
Cd 1	1.0 (<0.01)	1.8 (0.38)	1.0 (0.20)	1.2 (0.09)	1.1 (0.07)	1.0 (0.04)	1.3 (0.14)	1.3 (0.06)	0.8 (0.08)
Cd 2	2.6 (0.29)	2.5 (0.28)	1.9 (0.13)	2.0 (0.06)	2.3 (0.39)	1.7 (0.09)	2.1 (0.23)	2.5 (0.17)	1.7 (0.22)
Cd 3	3.4 (0.39)	3.4 (0.21)	2.8 (0.17)	3.4 (0.10)	3.4 (0.14)	2.1 (0.85)	3.5 (0.20)	4.0 (0.11)	2.9 (0.17)
Cd 4 <sup>c</sup>	3.8 (0.22)	4.7 (0.42)	3.3 (0.21)	4.6 (0.13)	4.2 (0.56)	3.7 (0.02)	4.1 (0.31)	4.8 (0.16)	3.5 (0.37)

<sup>a</sup> For abbreviations see Fig. 1.

<sup>b</sup> Values in parenthesis are standard error (n = 3).

<sup>c</sup> Values of Zn, Cu, and Cd for dose-response level 5 at SHI are 579 ± 20.3, 262 ± 12.6, and 5.0 ± 0.44 mg kg<sup>-1</sup>, respectively.

increasing the overall sample size, and can help reduce the 'noise' created by sampling error within each study (Hedges and Pigott, 2001).

Data was used from previously published results. Gibbs et al. (2006) report  $C_{mic}$  data for dose-response level 3 measured at each of the LTSE field sites during 1997. Soil microbial biomass data, across all dose-response levels, for years 1999, 2001, 2003, and 2005 was subsequently reported by Defra (2002, 2007a). All soil samples were collected to a depth of 25 cm during spring (April/May) with the exception of the 1997 sampling event for which samples were collected during autumn (October). In each case  $C_{mic}$  was determined using the method of chloroform (CHCl<sub>3</sub>) fumigation and potassium sulphate (K<sub>2</sub>SO<sub>4</sub>) extraction described by Vance et al. (1987). Samples of field moist soil were fumigated for 24 h using CHCl<sub>3</sub>. Organic C was extracted from fumigated and, duplicate, non-fumigated samples using 0.5 M K<sub>2</sub>SO<sub>4</sub> and subsequently determined by potassium dichromate oxidation (MAFF, 1986). Microbial biomass carbon was calculated as the difference in extracted C from fumigated and non-fumigated samples divided by the correction factor  $k_{EC} = 0.45$  (Jenkinson et al., 2004). The total concentrations of heavy metals within sludge amended soils were determined for each of the sampling events by aqua-regia acid digestion (McGrath and Cunliffe, 1985). The bioavailability of heavy metals was also monitored over the course of the LTSE by extracting exchangeable metal cations with ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>) as described by DIN (1997). Experimental data was reported as the arithmetic mean (x) of three replicates (n = 3) with standard error (SE); all standard errors were converted to standard deviations (SD = SE × √n) for the current investigation. Hence, the LTSE data set, as a whole, allows comparisons to be made between sites with a considerable degree of confidence, which would not necessarily be the case if a data set for meta-analysis was compiled from a number of independent, and international, sludge experiments with varying design.

For the purposes of meta-analysis an experimental treatment (E) must be compared to a suitable control (C). Microbial biomass carbon in soils receiving the Zn and Cd sludge treatments were compared to  $C_{mic}$  in soils receiving the uncontaminated digested sludge (Control 1; Table 4), whereas  $C_{mic}$  in soils receiving the Cu sludge treatment was compared to  $C_{mic}$  in soils receiving the uncontaminated undigested sludge (Control 2; Table 4). Each dose-response level (level 1–4, plus level 5 at SHI), at each of the nine LTSE field sites, was considered as an independent primary 'study', giving a total of  $k = 37$  studies for the three contaminated sludge treatments following each of the sampling events (except for 1997

where  $k = 9$ ). Total concentrations (mg kg<sup>-1</sup>) of Zn, Cu, and Cd were recorded as co-variables for each of the studies to allow comparison between the observed effect sizes, soil metal concentrations, and the UK statutory limits. A summary of all data included within the meta-analysis is given as supplementary information (Appendix 2).

### 2.3. Meta-analysis

All calculations were carried out according to Borenstein et al. (2009). Effects were calculated for each independent study as the 'log response ratio' (Hedges et al., 1999). A description of the effect size calculations is given as supplementary information (Appendix 3). Individual meta-analyses were carried out for each sludge treatment, grouping the data according to soil texture, cropping regime, and total metal concentration. The data for each sludge treatment was then grouped according to the date of each sampling event (1997, 1999, 2001, 2003, and 2005) to investigate the change in effect over time; a cumulative effect was also calculated by combining data for successive sampling events. Due to variations in soil texture, climate, land management practices, and discrepancies between the target metal concentrations and those actually achieved (Defra, 2002, 2007a, 2008), it could not be assumed that the overall effect on  $C_{mic}$  would be the same at each site; although the experimental design and applied sludge treatments were identical at each of the nine field sites. Therefore a 'random effect' model was used when calculating summary effects for the meta-analysis overall (Borenstein et al., 2009; Hedges and Vevea, 1998), with the data from each site considered to be an individual sample from a population of possible effects. This model assumes the true effect sizes for each individual study are normally distributed about a mean value and takes into account both sampling error *within* each study and the true variation in effects observed *between* sites. Calculated effect sizes represent the percentage difference in  $C_{mic}$  between soils receiving contaminated (Zn, Cu, Cd) sludge treatments in comparison to soils receiving uncontaminated (Controls 1 and 2) sludge treatments (Table 4). The effect size, 95% confidence limits, statistical significance, and number of studies are given for each group. Meta-analysis data is presented as 'forest plots', where each point represents the overall effect size for an individual group (Borenstein et al., 2009; Lewis and Clarke, 2001; Moja et al., 2007); for clarity only summary effect sizes are shown, effects calculated for individual studies are given as supplementary information (Appendix 2). The 95% confidence limits for each effect size are represented as horizontal lines; the observed effect is not statistically significant if the 95% confidence limit spans the centre line, at

which point the effect size is equal to 0 ( $\bar{x}_E = \bar{x}_C$ ).

### 3. Results

#### 3.1. Soil texture and cropping regime

##### 3.1.1. Zinc

With the exception of the clay loam soil at Pwllpeiran, where the effect of the Zn sludge treatment was least ( $-2.4\%$ ;  $CL_{95\%} = -10.3$  to  $6.1\%$ ;  $p = 0.569$ ;  $k = 4$ ), all of the effects observed when grouping the data by soil texture were negative and statistically significant. The Zn sludge treatment had the greatest impact on  $C_{mic}$  in the sandy loam soils at Watlington and Gleadthorpe ( $-16.9\%$ ;  $CL_{95\%} = -26.0$  to  $-6.7\%$ ;  $p < 0.01$ ;  $k = 8$ ), with a comparable effect also seen for the loamy sand soil of the Woburn site ( $-16.4\%$ ;  $CL_{95\%} = -25.8$  to  $-5.9\%$ ;  $p < 0.01$ ;  $k = 4$ ). However, no significant differences in effect were observed between different soil textures. Taken individually, the greatest effect size for the two sandy loam soils was seen at the Gleadthorpe site ( $-24.9\%$ ;  $CL_{95\%} = -38.0$  to  $-9.2\%$ ;  $p < 0.01$ ;  $k = 4$ ), whereas the magnitude of the effect size for the Watlington field site ( $-11.9\%$ ;  $CL_{95\%} = -23.8$  to  $2.0\%$ ;  $p = 0.090$ ;  $k = 4$ ) was non-significant, and lower in comparison to the Woburn site. The individual effect size for Watlington was also lower in comparison to the sandy clay loam soils of the Scottish field sites ( $-15.5\%$ ;  $CL_{95\%} = -24.1$  to  $-5.8\%$ ;  $p < 0.01$ ;  $k = 8$ ).

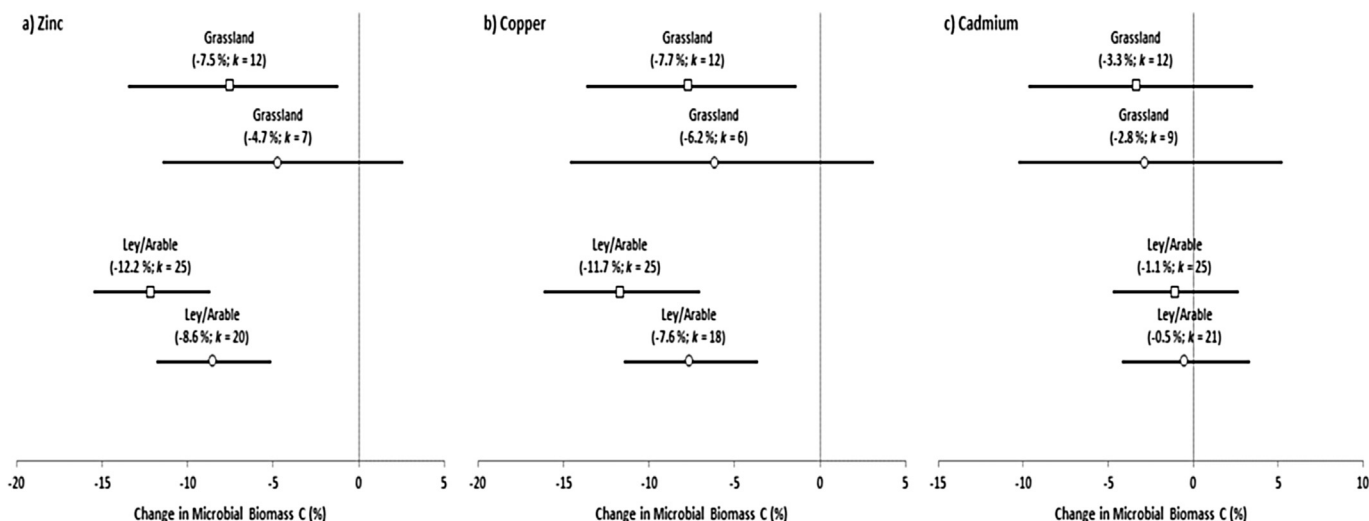
It is unclear why the Zn sludge treatment would have the greatest effect at the Gleadthorpe site. Gibbs et al. (2006) found that the bioavailability of Zn, i.e. the concentration of Zn extractable by  $NH_4NO_3$ , was inversely proportional ( $R^2 = 92\%$ ;  $p < 0.001$ ) to soil pH across the nine LTSE field sites in 1997. Though the only significant decrease in  $C_{mic}$  observed during 1997 was in soils receiving the Zn sludge treatment, at dose-response level 3, at the Gleadthorpe site. Of the nine field sites, soil pH was lowest at Hartwood (Table 2) and, over the course of the LTSE, concentrations of Zn extractable by  $NH_4NO_3$  were generally greater at Hartwood in comparison to the other field sites (Defra, 2002, 2007a; Gibbs et al., 2006). It would therefore be expected that the bioavailability of Zn, and the overall effect on  $C_{mic}$ , would be greatest at Hartwood. However, Gibbs et al. (2006) also observed that the amount of Cu extractable by  $NH_4NO_3$  was inversely proportional ( $p < 0.001$ ) to soil iron content, rather

than soil pH, accounting for 64% of the observed variation. It should be noted that the contaminated sludge used to prepare the Zn sludge treatment also contained concentrations of Cu significantly higher in comparison to Control 1 (Table 4). Since Gleadthorpe has the lowest iron content of the nine LTSE field sites (Table 2) it is possible that the magnitude of the observed effect is due to an additive impact of Zn and Cu on  $C_{mic}$  in soils receiving the Zn sludge treatment. The confounding influence of Cu in soils receiving the Zn sludge treatment is discussed below.

Application of the Zn sludge treatment to the three grassland sites caused an overall decrease in  $C_{mic}$  of 7.5% ( $CL_{95\%} = -13.5$  to  $-1.2\%$ ;  $p < 0.05$ ;  $k = 12$ ), whereas a decrease of 12.2% was observed for the ley/arable sites ( $CL_{95\%} = -15.5$  to  $-8.7\%$ ;  $p < 0.001$ ;  $k = 25$ ); in both cases the decreases were statistically significant (Fig. 2a). Removing data where the mean total metal concentration (over the course of the LTSE) exceeded the UK statutory limit for Zn (for the corresponding soil pH values at each site (Table 1), gave an overall effect size of  $-4.7\%$  ( $CL_{95\%} = -11.5$  to  $2.6\%$ ;  $p = 0.202$ ;  $k = 7$ ) for the grassland soils; though the effect was no longer statistically significant. For the ley/arable sites the overall effect remained statistically significant and indicated that  $C_{mic}$  had decreased by 8.6% ( $CL_{95\%} = -11.9$  to  $-5.1\%$ ;  $p < 0.001$ ;  $k = 20$ ) in soils where Zn was below the UK statutory limit (Fig. 2a). This suggests that  $C_{mic}$  may be more susceptible to heavy metal toxicity at the sites where a ley/arable cropping regime has been implemented. However it should be noted that no significant difference in effect was observed between the two types of field site.

##### 3.1.2. Copper

The effect of the Cu sludge treatment was also greatest for the sandy loam soils ( $-20.0\%$ ;  $CL_{95\%} = -27.5$  to  $-11.8\%$ ;  $p < 0.001$ ;  $k = 8$ ), with the magnitude of the effect slightly greater than that seen for Zn. Significant decreases of 16.0% and 15.2% were seen for  $C_{mic}$  in both sandy clay loam ( $CL_{95\%} = -26.0$  to  $-4.7\%$ ;  $p < 0.01$ ;  $k = 8$ ) and silty clay loam ( $CL_{95\%} = -20.9$  to  $9.0\%$ ;  $p < 0.001$ ;  $k = 8$ ) soils, respectively. Again, no significant differences in effect were observed between soil textures. The greatest individual effect size was seen at Gleadthorpe ( $-21.7\%$ ;  $CL_{95\%} = -31.9$  to  $-10.0\%$ ;  $p < 0.01$ ;  $k = 4$ ), with comparable effects also seen at Watlington ( $-20.6\%$ ;  $CL_{95\%} = -27.1$  to  $-13.5\%$ ;  $p < 0.001$ ;  $k = 4$ ) and Hartwood



**Fig. 2.** Forest plots showing effects of the a) Zn, b) Cu, and c) Cd sludge treatments on microbial biomass carbon (%) grouped according to the land management practices implemented at each field site. Each point represents the summary effect sizes determined by including (□) and excluding (○) data for soils where the mean total metal concentration during the LTSE exceeds the respective UK statutory limit. The number of primary studies ( $k$ ) used to calculate each effect size is indicated. Horizontal lines represent 95% confidence limits. Effects are not statistically significant if 95% confidence limit crosses the zero line.

(−19.2%;  $CL_{95\%} = -31.4$  to  $-4.9\%$ ;  $p < 0.05$ ;  $k = 4$ ). Above pH 5, Cu has been shown to have a high binding affinity to organic matter and is therefore readily immobilised within the soil environment (Alloway, 1995; McLaren and Crawford, 1973). For this reason the effect size observed at Hartwood appears to be anomalous as soil organic matter was greatest at the Hartwood site (Table 2). However, Gibbs et al. (2006) also found a positive relationship ( $p < 0.001$ ) between Cu extractable by  $NH_4NO_3$  and soil manganese content, which accounted for an additional 14% of the observed variation when included in the original regression model with soil iron content (see above). The greatest manganese content was seen at Hartwood (Table 2) which could possibly explain the observed effect size, if the presence of manganese offset the immobilisation of Cu by soil organic matter. The effect of the Cu sludge treatment in the loamy sand ( $-7.3\%$ ;  $CL_{95\%} = -18.2$  to  $5.0\%$ ;  $p = 0.233$ ;  $k = 4$ ) soil of the Woburn site was less than half of that observed for the Zn sludge treatment. Whereas the effects observed for the clay loam ( $-4.4\%$ ;  $CL_{95\%} = -11.6$  to  $3.4\%$ ;  $p = 0.260$ ;  $k = 4$ ) and calcareous clay loam ( $-3.3\%$ ;  $-13.4$  to  $8.0\%$ ;  $p = 0.555$ ;  $k = 4$ ) soils were non-significant and less than 5% in both cases.

For soils receiving the Cu sludge treatment, the overall decreases in  $C_{mic}$  observed at the grassland and ley/arable field sites were 7.7% ( $CL_{95\%} = -13.7$  to  $-1.4\%$ ;  $p < 0.05$ ;  $k = 12$ ) and 11.7% ( $CL_{95\%} = -16.1$  to  $-7.0\%$ ;  $p < 0.001$ ;  $k = 25$ ), respectively (Fig. 2b). In both cases the observed decreases were statistically significant and comparable to those seen in soils receiving the Zn sludge treatment. A significant decrease of 7.6% ( $CL_{95\%} = -11.5$  to  $-3.6\%$ ;  $p < 0.001$ ;  $k = 18$ ) was still observed for the ley/arable sites following the removal of data where the mean total concentration of Cu exceeded the UK statutory limit (Fig. 2b). Similarly, a decrease of 6.2% ( $CL_{95\%} = -14.6$  to  $3.1\%$ ;  $p = 0.187$ ;  $k = 6$ ) was observed for the grassland soils, though the effect was no longer significant. Below the UK statutory limit, it appears that application of the Cu sludge treatment may have caused similar decreases in  $C_{mic}$  regardless of the cropping regime implemented at each site. Though again no significant differences in effect were observed between the two types of field site.

### 3.1.3. Cadmium

Only in the calcareous clay loam soil did application of the Cd sludge treatment cause a significant decrease in  $C_{mic}$  above 5% ( $-6.6\%$ ;  $CL_{95\%} = -12.6$  to  $-0.2\%$ ;  $p < 0.05$ ;  $k = 5$ ); for the remaining

soils the observed effects were non-significant. No significant differences in effect were observed between soil textures.

The overall effects observed for the grassland and ley/arable field sites were  $-3.3\%$  ( $CL_{95\%} = -9.7$  to  $3.5\%$ ;  $p = 0.333$ ;  $k = 12$ ) and  $-1.1\%$  ( $CL_{95\%} = -4.7$  to  $2.7\%$ ;  $p = 0.575$ ;  $k = 25$ ), respectively (Fig. 2c). Little change in effect size was observed when data corresponding to a mean total Cd concentration above the UK statutory limit was removed from the analysis, with effects of  $-2.8\%$  ( $CL_{95\%} = -10.3$  to  $5.2\%$ ;  $p = 0.479$ ;  $k = 9$ ) and  $-0.5\%$  ( $CL_{95\%} = -4.2$  to  $3.3\%$ ;  $p = 0.794$ ;  $k = 21$ ) observed for the grassland and ley/arable sites, respectively (Fig. 2c). However, in neither case were the effects statistically significant, nor were any significant differences in effect observed between the two types of field site.

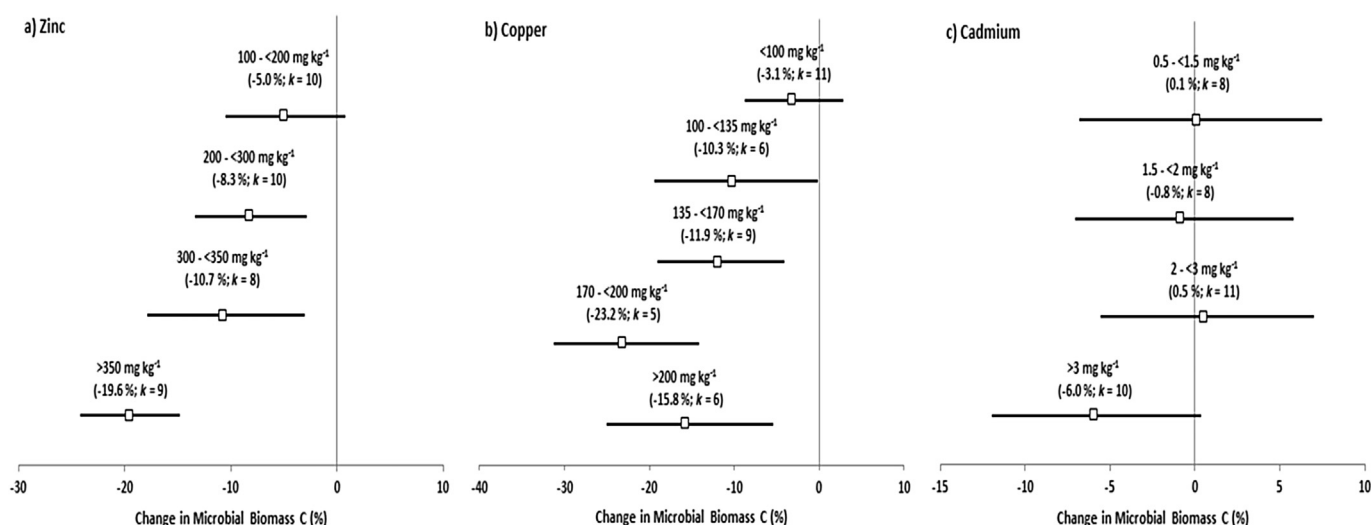
## 3.2. Total metal concentration

### 3.2.1. Zinc

A steady decline in  $C_{mic}$  was seen as the mean total metal concentration, over the course of the LTSE, increased in soils receiving the Zn sludge treatment (Fig. 3a). A significant decrease of 19.6% ( $CL_{95\%} = -24.1$  to  $-14.8\%$ ;  $p < 0.001$ ;  $k = 9$ ) was observed in soils where the mean total Zn exceeded  $350 \text{ mg kg}^{-1}$ . This was predominantly in soils receiving the Zn sludge treatment at dose-response level 4 and was significantly ( $p < 0.05$ ) greater in comparison to soils where total Zn fell below  $300 \text{ mg kg}^{-1}$ . Between 300 and  $350 \text{ mg kg}^{-1}$ , a significant decrease of 10.7% ( $CL_{95\%} = -17.8$  to  $-3.0\%$ ;  $p < 0.01$ ;  $k = 8$ ) was also observed. However more concerning is that where the mean total concentration of Zn fell below the current UK statutory limit, ranging between 200 and  $<300 \text{ mg kg}^{-1}$ ,  $C_{mic}$  decreased by approximately 8% ( $CL_{95\%} = -13.4$  to  $-2.8\%$ ;  $p < 0.01$ ;  $k = 10$ ). A non-significant decrease of 5% ( $CL_{95\%} = -10.5$  to  $0.8\%$ ;  $p = 0.090$ ;  $k = 10$ ) was also seen below  $200 \text{ mg kg}^{-1}$ , which has been suggested as an 'advisory limit' for Zn within sludge amended soils (MAFF/DoE, 1993b).

### 3.2.2. Copper

Similar to the Zn sludge treatment, a steady decline in  $C_{mic}$  was also observed as the mean total concentration of Cu increased (Fig. 3b). Above the UK statutory limit for Cu, significant decreases of 11.9% ( $CL_{95\%} = -19.4$  to  $-4.1\%$ ;  $p < 0.01$ ;  $k = 9$ ) and 23.2% ( $CL_{95\%} = -31.3$  to  $-14.1\%$ ;  $p < 0.001$ ;  $k = 5$ ) were observed for  $C_{mic}$



**Fig. 3.** Forest plots showing the effect of increasing total metal concentration ( $\text{mg kg}^{-1}$ ) on microbial biomass carbon (%) in soils receiving the a) Zn, b) Cu, and c) Cd sludge treatments. Each point ( $\square$ ) represents the summary effect size for data within specified concentration ranges. The number of primary studies ( $k$ ) used to calculate each effect size is indicated. Horizontal lines represent 95% confidence limits. Effects are not statistically significant if 95% confidence limit crosses the zero line.

in soils where mean total Cu ranged between 135 and  $<170 \text{ mg kg}^{-1}$  and 170 to  $<200 \text{ mg kg}^{-1}$ , respectively. Where total Cu ranged from 170 to  $<200 \text{ mg kg}^{-1}$  the decrease in  $C_{\text{mic}}$  was significantly ( $p < 0.05$ ) greater in comparison to soils where mean total Cu fell below  $100 \text{ mg kg}^{-1}$ . Above  $200 \text{ mg kg}^{-1}$ , the overall effect of Cu appeared to decrease ( $-15.8\%$ ;  $\text{CL}_{95\%} = -25.0$  to  $-5.4\%$ ;  $p < 0.01$ ;  $k = 6$ ), however in this case three of  $k = 6$  studies were from the calcareous clay loam and clay loam soils, for which the effect of Cu was least (see above). Decreases in  $C_{\text{mic}}$  were also observed in soils where values for mean total Cu fell below the UK statutory limit. A significant decrease of approximately 10% ( $\text{CL}_{95\%} = -19.3$  to  $-0.1\%$ ;  $p < 0.05$ ;  $k = 6$ ) was observed for soils where total Cu ranged from 100 to  $<135 \text{ mg kg}^{-1}$ , however below  $100 \text{ mg kg}^{-1}$  the effect was not significant ( $-3.1\%$ ;  $\text{CL}_{95\%} = -8.8$  to  $2.9\%$ ;  $p = 0.298$ ;  $k = 11$ ).

### 3.2.3. Cadmium

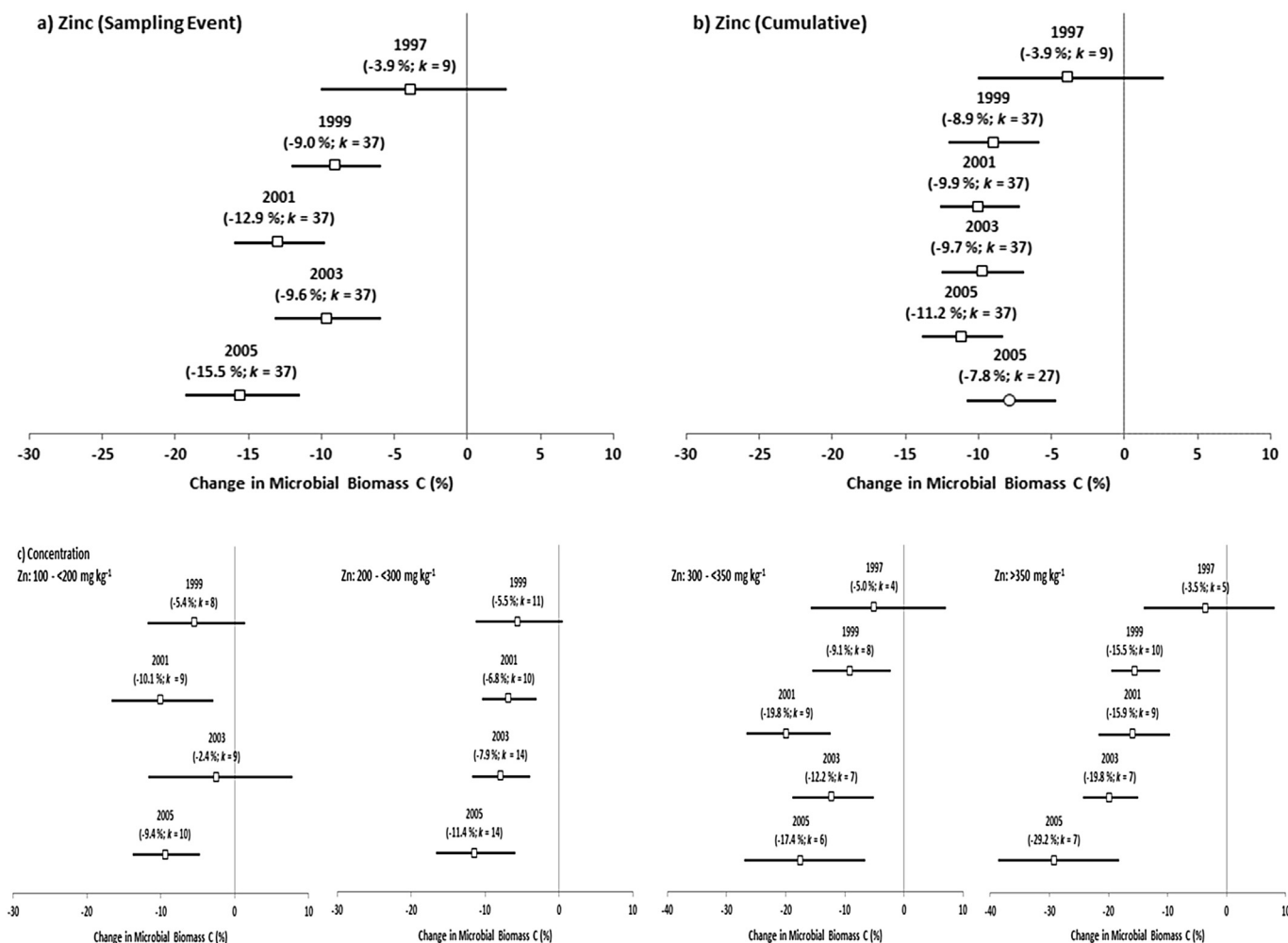
Although none of the observed effects were statistically significant, a marked increase in the magnitude of the effect size was observed for soils where total Cd exceeded the current UK statutory limit, indicating a 6.0% decrease in  $C_{\text{mic}}$  ( $\text{CL}_{95\%} = -11.9$  to  $0.4\%$ ;  $p = 0.065$ ;  $k = 10$ ). Nevertheless, below  $3 \text{ mg kg}^{-1}$  the Cd sludge

treatment appeared to have no overall effect on  $C_{\text{mic}}$ , with effect sizes ranging between  $\pm 1\%$  (Fig. 3c).

### 3.3. Change in effect over time

#### 3.3.1. Zinc

A significant ( $p < 0.05$ ) decline in  $C_{\text{mic}}$  was observed over the course of the LTSE for soils receiving the Zn sludge treatment (Fig. 4a). Soil microbial biomass decreased by 3.9% ( $\text{CL}_{95\%} = -10.0$  to  $2.3\%$ ;  $p = 0.245$ ;  $k = 9$ ) during 1997, at dose-response level 3, eventually reaching a 15.5% ( $\text{CL}_{95\%} = -19.3$  to  $-11.5\%$ ;  $p < 0.001$ ;  $k = 37$ ) decrease in 2005. With the exception of 2003, the effect of the Zn sludge treatment became increasingly negative for consecutive sampling events (Fig. 4a). The cumulative effect also shows a steady decline in  $C_{\text{mic}}$  over time, reaching an overall effect of  $-11.2\%$  ( $\text{CL}_{95\%} = -13.9$  to  $-8.3\%$ ;  $p < 0.001$ ;  $k = 37$ ) in 2005; although in this case the change in effect over time was not found to be statistically significant. Removing data where the total concentration of Zn exceeded the UK statutory limits caused a reduction in the overall cumulative effect. In this case a significant decrease of 7.8% ( $\text{CL}_{95\%} = -10.8$  to  $-4.7\%$ ;  $p < 0.001$ ;  $k = 27$ ) was observed, indicating that decreases in  $C_{\text{mic}}$  have occurred at concentrations below



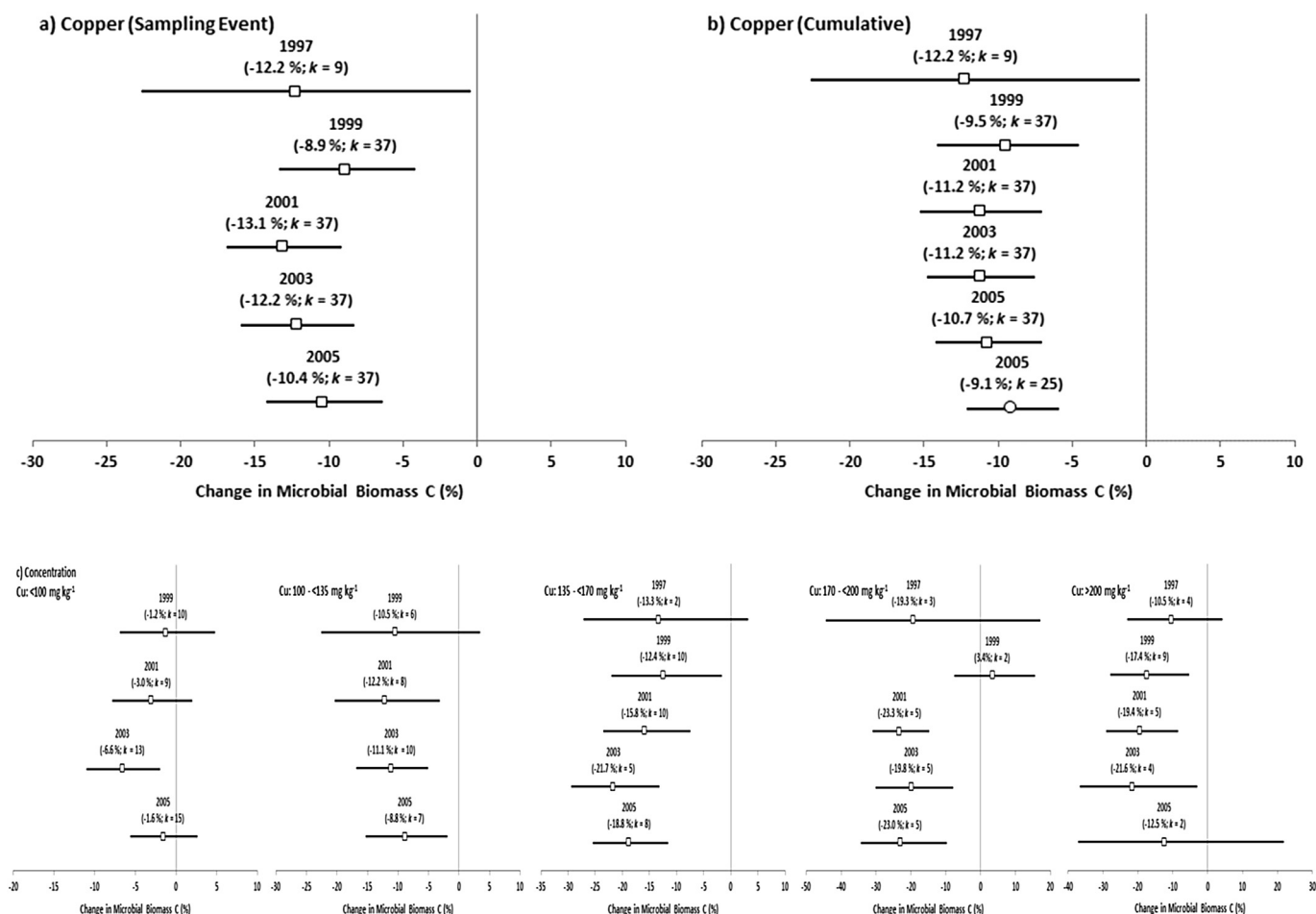
**Fig. 4.** Forest plots showing the change in effect of the Zn sludge treatment on microbial biomass carbon (%) over time; a) after each sampling event, b) cumulative effect over time, and c) the change in effect over time with increasing concentrations of total Zn. Each point ( $\square$ ) represents the summary effect size for specified sampling events, with the exception of the cumulative effect which represents the mean effect over consecutive years (A cumulative effect ( $\circ$ ) has also been calculated which excludes data for soils where the mean total metal concentration during the LTSE exceeds the respective UK statutory limit). The number of primary studies ( $k$ ) used to calculate each effect size is indicated. Horizontal lines represent 95% confidence limits. Effects are not statistically significant if 95% confidence limit crosses the zero line.



the UK statutory limit (Fig. 4b). In general, a decline in  $C_{mic}$  was seen over time at each level of the dose-response curve. Below  $200 \text{ mg kg}^{-1}$  the effect of the Zn sludge treatment fluctuated over the course of the LTSE with no significant change in  $C_{mic}$  observed over time (Fig. 4c); though significant decreases in  $C_{mic}$  were observed during 2001 ( $-10.1\%$ ;  $CL_{95\%} = -16.7$  to  $-2.9\%$ ;  $p < 0.05$ ;  $k = 9$ ) and 2005 ( $-9.4\%$ ;  $CL_{95\%} = -13.8$  to  $-4.7\%$ ;  $p < 0.05$ ;  $k = 10$ ). Where total Zn ranged between  $200$  and  $<300 \text{ mg kg}^{-1}$ , the change in effect over time resembled the cumulative effect (Fig. 4b and c). A non-significant effect of  $-5.5\%$  ( $CL_{95\%} = -11.2$  to  $0.5\%$ ;  $p = 0.073$ ;  $k = 11$ ) was observed in 1999, which increased to a significant effect of  $-11.4\%$  ( $CL_{95\%} = -16.6$  to  $-5.9\%$ ;  $p < 0.001$ ;  $k = 14$ ) in 2005; though no significant difference in effects were observed between the two sampling events. For soils where total Zn ranged from  $300$  to  $<350 \text{ mg kg}^{-1}$ , a non-significant decrease in  $C_{mic}$  of  $5\%$  ( $CL_{95\%} = -15.8$  to  $7.1\%$ ;  $p = 0.401$ ;  $k = 4$ ) was observed in 1997, whereas  $C_{mic}$  had decreased significantly by  $17.4\%$  ( $CL_{95\%} = -27.0$  to  $-6.6\%$ ;  $p < 0.01$ ;  $k = 6$ ) in 2005; though again the change in effect over time was not significant. Above  $350 \text{ mg kg}^{-1}$ , the effect size increased gradually for consecutive sampling events between 1999 and 2003 (Fig. 4c), before reaching an effect of  $-29.2\%$  ( $CL_{95\%} = -38.6$  to  $-18.3\%$ ;  $p < 0.001$ ;  $k = 7$ ) in 2005. In this case the decreases in  $C_{mic}$  observed in 2003 and 2005 were significantly ( $p < 0.05$ ) greater in comparison to those observed in 1997.

### 3.3.2. Copper

Application of the Cu sludge treatment appears to have caused a persistent decrease in  $C_{mic}$  in the receiving soils with significant and negative effects seen for each of the sampling events over the course of the LTSE (Fig. 5a). This is also indicated by the cumulative effect, which remained at approximately  $-11\%$  from 2001 onwards. Removing data where the total concentration of Cu exceeds the UK limit caused little change in the overall cumulative effect, which remained close to  $10\%$  ( $-9.1\%$ ;  $CL_{95\%} = -12.1$  to  $-5.9\%$ ;  $p < 0.001$ ;  $k = 25$ ) in 2005, again indicating significant decreases in  $C_{mic}$  have occurred at concentrations below the UK statutory limit (Fig. 5b). However, the overall effects for the individual sampling events suggest  $C_{mic}$  may be recovering in the contaminated soils (Fig. 5a), as the effect size changed from  $-13.1\%$  ( $CL_{95\%} = -16.9$  to  $-9.2\%$ ;  $p < 0.001$ ;  $k = 37$ ) in 2001 to  $-10.4\%$  ( $CL_{95\%} = -16.9$  to  $-9.2\%$ ;  $p < 0.001$ ;  $k = 37$ ); though the change was not statistically significant. This was predominantly seen in soils where the total concentration of Cu fell below  $170 \text{ mg kg}^{-1}$  (Fig. 5c). Below  $100 \text{ mg kg}^{-1}$ , a significant effect size of  $-6.6\%$  ( $CL_{95\%} = -11.0$  to  $-1.9\%$ ;  $p < 0.01$ ;  $k = 13$ ) was observed in 2003. However, by 2005 the observed effect was only  $-1.6\%$  ( $CL_{95\%} = -5.6$  to  $2.6\%$ ;  $p = 0.459$ ;  $k = 15$ ), comparable to the initial effect seen in 1999. This could indicate the recovery of  $C_{mic}$  over time; however no significant differences between effects were observed for the two



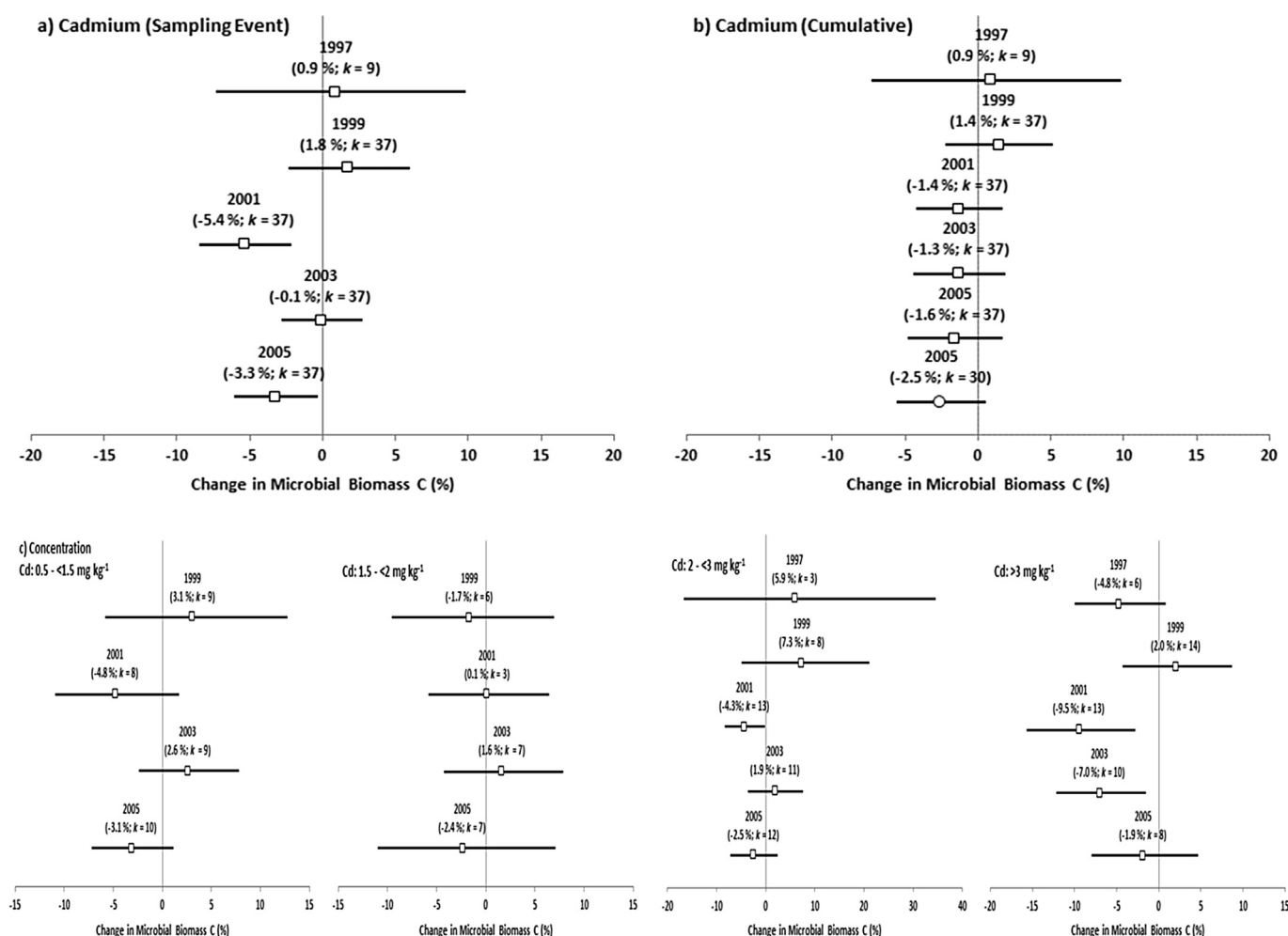
**Fig. 5.** Forest plots showing the change in effect of the Cu sludge treatment on microbial biomass carbon (%) over time; a) after each sampling event, b) cumulative effect over time, and c) the change in effect over time with increasing concentrations of total Cu. Each point (□) represents the summary effect size for specified sampling events, with the exception of the cumulative effect which represents the mean effect over consecutive years (A cumulative effect (○) has also been calculated which excludes data for soils where the mean total metal concentration during the LTSE exceeds the respective UK statutory limit). The number of primary studies ( $k$ ) used to calculate each effect size is indicated. Horizontal lines represent 95% confidence limits. Effects are not statistically significant if 95% confidence limit crosses the zero line.

sampling events. Similarly, where total Cu ranged from 100 to  $<135 \text{ mg kg}^{-1}$ , a significant decrease of 12.2% ( $\text{CL}_{95\%} = -20.4$  to  $-3.1\%$ ;  $p < 0.01$ ;  $k = 8$ ) was observed in 2001. A gradual decrease in effect size can then be seen for the remainder of the LTSE, with an 8.8% ( $\text{CL}_{95\%} = -15.3$  to  $-1.9\%$ ;  $p < 0.05$ ;  $k = 7$ ) decrease in  $C_{\text{mic}}$  observed in 2005; though again no significant change in effect was observed over time. Therefore, although significant decreases in  $C_{\text{mic}}$  have occurred at concentrations below the UK statutory limit, the effect does not appear to be long-term with  $C_{\text{mic}}$  showing signs of recovery within 4–6 years of the final sludge application. Where total Cu ranged between 135 and  $<170 \text{ mg kg}^{-1}$ ,  $C_{\text{mic}}$  gradually declined over the course of the LTSE with a 21.7% ( $\text{CL}_{95\%} = -29.4$  to  $-13.2\%$ ;  $p < 0.001$ ;  $k = 5$ ) decrease observed in 2003. Again, although the change in effect is not statistically significant, the decrease in  $C_{\text{mic}}$  ( $-18.8\%$ ;  $\text{CL}_{95\%} = -25.4$  to  $-11.5\%$ ;  $p < 0.001$ ) observed in 2005 was lower than for the previous sampling event, but remained more than twice that seen for soils where total Cu concentration fell below the UK limit (Fig. 5c). For soils where the total concentration of Cu exceeded  $170 \text{ mg kg}^{-1}$  the effect of the Cu sludge treatment appeared to be more lasting. Where total Cu ranged from 170 to  $<200 \text{ mg kg}^{-1}$  no significant change in the effect size was seen from 2001 to 2005, with significant decreases in  $C_{\text{mic}}$  of approximately 23% seen for both years (Fig. 5c). Due to a low

sample size of  $k = 2$  studies an anomalous increase in  $C_{\text{mic}}$  appears to have occurred following the 1999 sampling event, which should be considered with caution; this is likely to be due to the minor effect of the Cu sludge treatment observed for the clay loam soil. Where the total concentration of Cu exceeded  $200 \text{ mg kg}^{-1}$  an increasingly negative effect size was observed over the course of the LTSE, changing from  $-10.5\%$  in 1997 ( $\text{CL}_{95\%} = -23.0$  to  $4.2\%$ ;  $p < 0.05$ ;  $k = 4$ ) to  $-21.6\%$  ( $\text{CL}_{95\%} = -36.7$  to  $-2.9\%$ ;  $p < 0.05$ ;  $k = 4$ ) in 2003. However, although increasingly greater and statistically significant decreases in  $C_{\text{mic}}$  were observed between 1999 and 2003, the change in effect over time was not found to be statistically significant (Fig. 5c).

### 3.3.3. Cadmium

The Cd sludge treatment appears to have had little effect on  $C_{\text{mic}}$  over the course of the LTSE, though significant effects of  $-5.4\%$  ( $\text{CL}_{95\%} = -8.5$  to  $-2.2\%$ ;  $p < 0.01$ ;  $k = 37$ ) and  $-3.3\%$  ( $\text{CL}_{95\%} = -6.1$  to  $-0.3\%$ ;  $p < 0.05$ ;  $k = 37$ ) were seen for the 2001 and 2005 sampling events, respectively (Fig. 6a). The cumulative effect over time showed no significant effect over the course of the LTSE reaching an overall non-significant effect of  $-1.6\%$  ( $\text{CL}_{95\%} = -4.8$  to  $1.7\%$ ;  $p = 0.338$ ;  $k = 37$ ) in 2005. Removing data where Cd exceeded the UK statutory limit increased the magnitude of the cumulative



**Fig. 6.** Forest plots showing the change in effect of the Cd sludge treatment on microbial biomass carbon (%) over time; a) after each sampling event, b) cumulative effect over time, and c) the change in effect over time with increasing concentrations of total Cd. Each point ( $\square$ ) represents the summary effect size for specified sampling events, with the exception of the cumulative effect which represents the mean effect over consecutive years (A cumulative effect ( $\circ$ ) has also been calculated which excludes data for soils where the mean total metal concentration during the LTSE exceeds the respective UK statutory limit). The number of primary studies ( $k$ ) used to calculate each effect size is indicated. Horizontal lines represent 95% confidence limits. Effects are not statistically significant if 95% confidence limit crosses the zero line.

effect seen in 2005 to  $-2.5\%$  ( $CL_{95\%} = -5.6$  to  $0.6\%$ ;  $p = 0.109$ ;  $k = 30$ ), though the effect remained non-significant (Fig. 6b). Below  $2 \text{ mg kg}^{-1}$ , the effect of the Cd sludge treatment did not exceed  $\pm 5\%$ , nor were any significant effects or changes over time observed (Fig. 6c). For soils where total Cd fell below the UK statutory limit,  $2$  to  $<3 \text{ mg kg}^{-1}$ , a significant effect of  $-4.3\%$  ( $CL_{95\%} = -8.3$  to  $-0.2\%$ ;  $p < 0.05$ ;  $k = 13$ ) was observed in 2001. Above  $3 \text{ mg kg}^{-1}$ , significant decreases in  $C_{mic}$  of  $9.5\%$  ( $CL_{95\%} = -15.6$  to  $-2.8\%$ ;  $p < 0.01$ ;  $k = 13$ ) and  $7.0\%$  ( $CL_{95\%} = -12.2$  to  $-1.5\%$ ;  $p < 0.05$ ;  $k = 10$ ) were seen for years 2001 and 2003, respectively. However, by 2005 the observed effect was  $-1.9\%$  ( $CL_{95\%} = -8.0$  to  $4.6\%$ ;  $p = 0.981$ ;  $k = 8$ ) and was no longer statistically significant. In neither case were the changes observed over time found to be statistically significant.

#### 4. Discussion

A statistical review of the LTSE experimental data has previously been commissioned by Defra. Repeated measure ANOVA and multiple-regression were used to combine experimental data for each of the LTSE field sites into a single analysis (Defra, 2008). In this case a significant experimental confound was identified which affected data interpretation. The sludge used to produce the Zn experimental treatment also contained significantly higher concentrations of Cu in comparison to the uncontaminated Control 1, whereas the Cd sludge treatment also contained significantly higher concentrations of Zn (Table 4; Defra, 2008). Given that the Cu sludge treatment was least affected by confounding metal contamination, the effect of Cu on  $C_{mic}$  was determined first and then used to 'adjust' the data by adding  $-m \times \ln([Cu])$  to the values of  $C_{mic}$  observed in soils receiving the Zn and Cd sludge treatments, where  $m$  is the slope of regression between  $\ln([Cu])$  and  $C_{mic}$  and  $[Cu]$  is the total concentration of Cu in a given plot (Defra, 2008).

##### 4.1. Zinc

Adjusting for the concentration of Cu in soils receiving the Zn sludge treatment Defra (2008) observed a decrease in  $C_{mic}$  of  $6.2\%$  as total Zn increased from background levels to the statutory limit. Which is in agreement with the overall cumulative effect determined when data above the statutory limit were excluded from the analysis (Fig. 4b). However, the decrease in  $C_{mic}$  determined by Defra (2008) is lower in comparison to those observed during the current investigation for soils where total Zn ranged from  $200$  to  $<300 \text{ mg kg}^{-1}$  and  $300$  to  $<350 \text{ mg kg}^{-1}$  (Fig. 3a). Over the course of the LTSE the total concentration of Cu in these soils ranged from  $50.6$  to  $90.8 \text{ mg kg}^{-1}$  (Appendix 2), with only two outliers above  $100 \text{ mg kg}^{-1}$ . Hence, assuming an individual effect for each metal, the contribution of Cu to the overall effect in these soils, based on the results described above, is expected to be around  $-3\%$  (Fig. 3b), which would suggest the individual effect of Zn within these soils is approximately  $-5$  and  $-8\%$ , respectively. A significant interaction between the effects of Zn and Cu was observed during the Defra analysis indicating that the effects of Zn and Cu on  $C_{mic}$  were additive and may be more harmful to microorganisms when present simultaneously (Defra, 2008). This has also been suggested by Chander and Brookes (1993), who saw greater decreases in  $C_{mic}$  when Zn and Cu were applied in combination. A decrease of  $6\%$  was observed in soils where the total concentration of Zn was  $375 \text{ mg kg}^{-1}$ , whereas a  $23\%$  decrease in  $C_{mic}$  was observed when both Zn and Cu were present simultaneously at concentrations of  $322$  and  $176 \text{ mg kg}^{-1}$ , respectively (Chander and Brookes, 1993); a decrease also greater than that seen for soils containing Cu at  $197 \text{ mg kg}^{-1}$  (see below). For the LTSE soils where total Zn exceeded  $350 \text{ mg kg}^{-1}$ , the confounding Cu contamination ranged from  $80.2$  to  $117.8 \text{ mg kg}^{-1}$ , with one outlier below  $80 \text{ mg kg}^{-1}$  (Appendix 2).

In this case a comparable decrease in  $C_{mic}$  of approximately  $20\%$  was observed (Fig. 3a), although the concentrations of Zn and Cu present within the LTSE soils were higher, and lower, respectively, in comparison those reported by Chander and Brookes (1993). Khan and Scullion (1999) have also observed a significant interaction between the effects of Zn and Cu on  $C_{mic}$  in soils receiving a 'Zn + Cu' sludge treatment (spiked with metal salts) which had been incubated for three weeks. However, despite significant decreases in  $C_{mic}$ , no significant interaction in effect was observed for the two metals after a period of 7 weeks.

It has often been assumed that the bioavailability of a metal in sludge amended soils will increase linearly in proportion to total concentration that is applied (McBride, 1995). However, this will not be the case if heavy metals interact and compete with each other for binding sites within the soil environment. Significant interactions between the concentration of Zn extractable by  $\text{CaCl}_2$  and the total concentration of Cu were also observed by Khan and Scullion (1999) indicating that the presence of one metal may influence the bioavailability of the other. However, after a period of 7 weeks the concentration of Zn extractable by  $\text{CaCl}_2$  appeared to decrease in soils where total Cu was approximately  $182 \text{ mg kg}^{-1}$ . Therefore it may be that longer periods of time are necessary before the additive toxic effect of Zn and Cu manifests in sludge amended soils. Kim and McBride (2009) investigated the interactions between Zn and Cu on the concentrations of each metal extractable by  $\text{CaCl}_2$  as the total concentration of both metals were increased in combinations from  $0$  to  $400 \text{ mg kg}^{-1}$ . In this case two soil types, a sandy loam and a silty clay loam, were spiked with metal salts and allowed to equilibrate under field conditions for a year. For the sandy loam soil, increases of  $48.5$  and  $26.8\%$  (in comparison to respective control soils with no added Zn or Cu) were seen for the extractability of Zn and Cu, respectively, at total metal concentrations of  $400 \text{ mg Zn kg}^{-1}$  and  $100 \text{ mg Cu kg}^{-1}$ . However, at a total Cu concentration of  $50 \text{ mg kg}^{-1}$ , and  $400 \text{ mg Zn kg}^{-1}$ , extractable Zn decreased by  $16\%$  whereas the increase in Cu extractability was  $6.5\%$ . At  $200 \text{ mg Zn kg}^{-1}$ ,  $24.3$  and  $11.0\%$  increases in extractable Zn were observed when Cu was present at total concentrations of  $50$  and  $100 \text{ mg Cu kg}^{-1}$ , respectively; extractable Cu also increased by  $29.7$  and  $36.5\%$  for the respective concentrations of Cu. In the silty clay loam soil, the extractability of Zn increased by  $9$  and  $8\%$ , respectively, when Zn was present at a total concentration of  $200 \text{ mg kg}^{-1}$  and Cu present at  $50$  and  $100 \text{ mg kg}^{-1}$ . However, only at a total concentration of  $50 \text{ mg Cu kg}^{-1}$  was an increase in extractable Cu (of  $27.3\%$ ) observed; at  $100 \text{ mg Cu kg}^{-1}$ , Cu extractability appeared to decrease by  $2.7\%$ . At a total Zn concentration of  $400 \text{ mg kg}^{-1}$ , increases in the extractability of Zn and Cu of  $5.4\%$  and  $31.0\%$ , respectively, were observed at  $50 \text{ mg Cu kg}^{-1}$ , whereas at  $100 \text{ mg Cu kg}^{-1}$ , the extractability of Cu increased by  $26.8\%$  while that for Zn appeared to decrease by  $7.4\%$ .

The augmentation of Zn and Cu bioavailability, when both metals are present simultaneously, could possibly explain the large effect size observed for the sandy loam soils (Gleadthorpe and Watlington), as well as the observed decline in  $C_{mic}$  over time. Changes in the concentration of Zn and Cu extractable by  $\text{NH}_4\text{NO}_3$ , over the course of the LTSE, are presented as supplementary information for soils receiving the Zn sludge treatment at each of the nine field sites (Appendix 4). It should be noted that  $\text{NH}_4\text{NO}_3$ , rather than  $\text{CaCl}_2$ , was used to determine the bioavailability of Zn and Cu throughout the LTSE, however, Pueyo et al. (2004) have demonstrated that both methods are in agreement. At the Gleadthorpe site the concentration of Zn extractable by  $\text{NH}_4\text{NO}_3$  at dose-response levels 2–4, increased following the final sludge applications, reaching a plateau around the 2001 sampling event. Whereas little change in the extractability of Cu was observed. Hence the effect at Gleadthorpe may be due to an increase in the

bioavailability of Zn in the presence of Cu, rather than an increase in the solubility of Cu, due to the inverse relation to soil iron content mentioned previously (Gibbs et al., 2006). A contrasting interaction appears to have occurred at the Watlington site, with the bioavailability of Zn decreasing over the course of the LTSE while that of Cu has increased (Appendix 4). This trend could potentially explain the continued decline in  $C_{mic}$  observed over the course of the LTSE in soils receiving the Zn sludge treatment, as the exposure to bioavailable metal contamination has been sustained. With the exception of the Shirburn site, the bioavailability of Zn generally reached a maximum at all of the LTSE field sites around the 2001 sampling event, before steadily declining; except in the case of Gleadthorpe. Subsequent increases in the bioavailability of the confounding Cu contamination were also seen at Pwllpeiran, Shirburn, and Woburn.

#### 4.2. Copper

In agreement with the overall effect seen for the sandy loam soils of the LTSE, Chander and Brookes (1991) observed a decrease in  $C_{mic}$  of 18% in a sandy loam soil at the Luddington Experimental Station (Warwickshire, UK), receiving applications of a 'Low-Cu' sludge treatment over the course of 4 years (1968–1972); almost 20 years prior to the investigation. In this case the total concentration of Cu present within the Luddington soil was  $150 \text{ mg kg}^{-1}$ , therefore the observed decrease in  $C_{mic}$  is greater in comparison to that seen for the LTSE soils where total Cu ranged from  $135$  to  $<170 \text{ mg kg}^{-1}$  (Fig. 3b). In contrast, the 'Low-Cu' sludge treatment appeared to have no effect on  $C_{mic}$  when applied to a silty loam soil at the Lee Valley Experimental Station (Hertfordshire, UK), despite a total Cu concentration of  $212 \text{ mg kg}^{-1}$ . This discrepancy was thought to be due to the higher clay and organic matter content of the Lee Valley soil (Chander and Brookes, 1991). A 15.2% decrease in  $C_{mic}$  was observed overall for the silty clay loam soils at Bridgets and Rosemaund, for which the clay contents were comparable to that of Lee Valley (21%), however organic matter at Lee Valley was approximately twice that of the two LTSE field sites (Table 2). A 40% decrease in  $C_{mic}$  was observed at both Luddington and Lee Valley sites following the application of a 'High-Cu' sludge treatment, which increased the total concentration of Cu above  $350 \text{ mg kg}^{-1}$  in both cases. The highest concentration of Cu measured during the LTSE was  $258.5 \text{ mg kg}^{-1}$ , seen at the Auchincruive site in 2003 (Appendix 2), hence such a decrease in  $C_{mic}$  is not expected for any of the LTSE sites. Chander and Brookes (1993) also observed an 18% decrease in  $C_{mic}$  at the Gleadthorpe Experimental Husbandry Farm (Nottinghamshire, UK), in soils receiving two applications of a Cu sludge treatment (1982 and 1986). Samples were collected 4 years following the final application of sludge at which point the total concentration of Cu was  $197 \text{ mg kg}^{-1}$ . Again this is in agreement with the overall effect determined for the sandy loam soils, and the individual effect determined for the Gleadthorpe site. In this case the decrease in  $C_{mic}$  observed by Chander and Brookes (1993) falls between that observed for LTSE soils where total Cu ranged between  $170$  and  $<200 \text{ mg kg}^{-1}$ , or exceeded  $200 \text{ mg kg}^{-1}$  (Fig. 3b).

An 18% decrease in  $C_{mic}$  was also observed by Defra (2008) in soils receiving the Cu sludge treatment, as total Cu increased from background levels to the UK statutory limit. This value is higher than the effect observed during the current analysis for soils where mean total Cu ranged from  $100$  to  $<135 \text{ mg kg}^{-1}$  (Fig. 3b). However, this discrepancy may be due to the omission of data from the Shirburn site in the Defra analysis. The effect of the Cu sludge treatment was least in the calcareous clay loam soil, therefore including this site in the current analysis will have reduced the overall effect size for groups containing data from Shirburn. However, it is unclear why the Cu sludge treatment would have such

little effect at this site. Despite both a high pH and soil organic matter content at the Shirburn site (Table 2), the concentrations of Cu extractable by  $\text{NH}_4\text{NO}_3$ , were amongst the highest of the nine LTSE field sites (Gibbs et al., 2006; Defra, 2002, 2007a). As mentioned previously, Cu is readily immobilised by organic matter within the soil environment, hence a reduction in the bioavailability of Cu would be expected for this soil. Similarly, for the clay loam soil at Pwllpeiran, which had a greater organic matter content in comparison to Shirburn, as well as one of the highest iron contents of the nine field sites (Table 2), it would be expected that the effect of the Cu sludge treatment would be low at this site. However, an increase in the concentration of Cu extractable by  $\text{NH}_4\text{NO}_3$  was observed over the course of the LTSE (data not shown). This was also observed at the Watlington field site (data not shown) and is in agreement with the trends observed for the confounding Cu contamination in soils receiving the Zn sludge treatment (Appendix 4). In general, the values for the concentration of Cu extractable by  $\text{NH}_4\text{NO}_3$  remained stable over the course of the LTSE, following a sharp decrease between 1997 and 1999 in some cases (Gibbs et al., 2006; Defra, 2002, 2007a). Hence, the apparent recovery of  $C_{mic}$  observed following the 2001 sampling event (Fig. 5a and c) may indicate the microbial population has become tolerant to the presence of Cu, particularly if the bioavailability of Cu appears to have increased at some of the field sites.

#### 4.3. Cadmium

Data for soils receiving the Cd sludge treatment were also adjusted by Defra (2008) to account for the presence of Cu. In this case  $C_{mic}$  was seen to decrease by 3.4%, however a stronger relationship was observed between  $C_{mic}$  and the total concentration of Zn rather than Cd (Defra, 2008). The results presented here suggest the Cd sludge treatment has had no effect on  $C_{mic}$  where the mean total concentration of Cd was below  $3 \text{ mg kg}^{-1}$ , though a non-significant decrease of 6% was observed above this limit (Fig. 3c). For soils where total Cd exceeded  $3 \text{ mg kg}^{-1}$  the concentrations of Zn and Cu ranged from  $98.3$  to  $230 \text{ mg kg}^{-1}$  (with two outliers above  $250 \text{ mg kg}^{-1}$  seen at Shirburn) and  $45.5$  to  $87.1 \text{ mg kg}^{-1}$ , respectively (Appendix 2). Hence these soils, predominantly receiving the Cd sludge treatment at dose-response level 4, are largely comparable to soils receiving the Zn sludge treatment at dose response levels 1 and 2. Here the observed effect falls between those observed for soils in which mean total Zn ranged from  $100$  to  $<200 \text{ mg kg}^{-1}$ , and  $200$  to  $<300 \text{ mg kg}^{-1}$  (Fig. 3a). This could also explain the apparently anomalous increase in the cumulative effect observed when data for soils exceeding the UK statutory limit for Cd was excluded from the meta-analysis (Fig. 6b), as little change in Zn concentration was seen ( $85.0$ – $213 \text{ mg kg}^{-1}$  to  $85.0$ – $173.4 \text{ mg kg}^{-1}$ ). However, the concentrations of Zn extractable by  $\text{NH}_4\text{NO}_3$  were generally lower for soils receiving the Cd sludge treatment, at each level of the dose response curve, in comparison to soils receiving the Zn sludge treatment at dose-response level 1, and considerably lower than dose response level 2 (Gibbs et al., 2006; Defra, 2002, 2007a). Furthermore, the bioavailability of both Zn and Cd appeared to be least at the Shirburn site, with concentrations of Zn and Cd extractable by  $\text{NH}_4\text{NO}_3$  below  $0.15$  and  $0.03 \text{ mg kg}^{-1}$ , respectively, for the duration of the LTSE (data not shown). Yet the greatest effect for soil receiving the Cd sludge treatment was observed at Shirburn. Hence it cannot be said for certain if either metal is having an effect on  $C_{mic}$  in these soils. This also raises the question of whether the Cd sludge treatment contained organic or inorganic components which could have immobilised Zn more readily in comparison to the Zn sludge treatment.

Significant decreases in  $C_{mic}$  have been reported in soils heavily



contaminated with Cd. For instance, Brookes and McGrath (1984) observed decreases in  $C_{mic}$  of up to 50% at the Woburn Market Garden Experiment (Bedfordshire, UK) in soils receiving sewage sludge applications over the course of 20 years (1942–1960). In this case concentrations of Zn, Cu, and Cd reached 340, 125.4, and 8.8 mg kg<sup>-1</sup>, respectively, following the final application of sludge in 1960 (McGrath, 1984). A follow up investigation by Abaye et al. (2005) also reported a 20% decrease in  $C_{mic}$  where total Cd was twice the UK limit (6.0–6.3 mg kg<sup>-1</sup>); concentrations of Zn and Cu ranged from 212.3 to 230.1 and 67.0–82.6 mg kg<sup>-1</sup>, respectively. The highest concentration of Cd measured during the LTSE was 4.7 mg kg<sup>-1</sup>; seen at the Hartwood field site in 2001 (Appendix 2). Hence these results describe more extreme cases of contamination in comparison to those of the current investigation. However, there does not appear to be anything within the current data-set to suggest that the level of Cd contamination present at the LTSE field sites has had an observable effect on  $C_{mic}$ . This would suggest that the current UK limit for Cd is sufficient to protect  $C_{mic}$  from Cd toxicity.

#### 4.4. Practical significance

Soil microbial biomass C typically ranges from 200 to 1000 mg C<sub>mic</sub> kg<sup>-1</sup> in agricultural soils (Insam and Parkinson, 1989; Martens, 1995), accounting for approximately 1–3% of soil organic carbon (Jenkinson and Ladd, 1981). Measurements of  $C_{mic}$  have been used to monitor long-term changes in soil organic C and the incorporation of fresh organic matter into the soil environment (Powelson and Brookes, 1987). However, despite significant improvements in the methods for quantifying  $C_{mic}$ , Stockdale and Brookes (2006) suggest that gross measurements of  $C_{mic}$  have had little influence on agricultural practice in general. Hence two questions have been posed when considering the potential impact of sewage sludge applications on soil microorganisms, these are: a) ‘do microbes matter?’ (Giller et al., 1999) and, if so, b) ‘where’s the limit?’ (Dahlin et al., 1997). The approach to regulating sewage sludge applications to agricultural land varies considerably between EU member states, with the UK statutory limits for Zn, Cu, and Cd, amongst, if not the highest in Europe (McGrath et al., 1994). The results presented here show that significant decreases in  $C_{mic}$  have occurred in soils where the total concentrations of Zn and Cu fall below the current UK statutory limits; ranging from 6.8 to 11.4% and 6.6 to 12.2%, respectively, depending on how the data is grouped. A continued decline in  $C_{mic}$  could be seen in soils with elevated concentrations of both Zn and Cu (Fig. 4c), whereas in the case of Cu alone, the effect does not appear to be lasting, with  $C_{mic}$  showing signs of recovery after a period of 6 years (Fig. 5c). In addition, the results presented here indicate that  $C_{mic}$  may be more susceptible to heavy metal toxicity in the soils where a ley/arable cropping regime has been implemented. However this may simply be due to there being a greater number of ley/arable field sites, allowing a more accurate estimation of the effect size to be calculated.

Soil microbial biomass and microbial community structures are influenced by the indigenous soil properties as well as the land management practices experienced by the soil (Schutter et al., 2001; Wu et al., 2012). Hence, with regards to the LTSE, despite the same contaminated sludge treatments being applied at each site, it cannot be assumed that the response of the microbial communities would be the same for each of the nine LTSE field sites; for this reason a random effects model was chosen for the meta-analysis. The varied response of microorganisms within agricultural soils is often attributed to the varying metal content of the applied sludge, however Chander et al. (2001) have suggested that other environmental factors need to be considered, such as the nature and quantity of the C input associated with the applied

sludge. Similarly, Anderson et al. (2009) suggest that the cumulative influence of environmental factors is more likely to affect the microbial response rather than the nature of an environmental stress, such as heavy metal contamination. The apparent differences in the effect of the Zn and Cu sludge treatments on  $C_{mic}$  observed over time, may be due in part to the differing nature of the digested and undigested organic C that was applied (Table 4), and the capacity for the soil microbial community to utilise it as a food source. For instance, with the exception of Pwllpeiran, greater increases in  $C_{mic}$ , in comparison to untreated soil, were seen for soils receiving the Cu sludge treatment in 1997, in comparison to soils receiving the Zn treatment (Gibbs et al., 2006). However given that the analysis of  $C_{mic}$  is a gross measurement of microbial community size it cannot be said whether the microbial biomass measured at the end of the LTSE is comprised of the same species present prior to the application of sludge, or even those present following the final applications of sludge in 1997. Many soil biological indicators detect changes in microbial activity while  $C_{mic}$  remains constant, therefore the observation of significant decreases in  $C_{mic}$  often indicates major changes in microbial community structure (Campbell, Personal Communication). This is cause for concern particularly if the reduction in  $C_{mic}$  corresponds to the loss of microbial species responsible for essential soil functions.

The extent to which a soil can tolerate a decrease in  $C_{mic}$  without losing essential soil functions is still not fully understood (Brooks et al., 2007; Singh et al., 2014), and it may be that a small decrease in  $C_{mic}$  corresponds to a drastic decrease in soil fertility if an essential species of microorganism is lost (Chaudri et al., 2008; Singh et al., 2014). Macdonald et al. (2011) have investigated changes in the microbial community structure for soils receiving the Zn and Cu sludge treatments (at dose-response levels 2–4), at seven of the LTSE field sites (Pwllpeiran and Shirburn were not included). Genomic analysis of the contaminated soils showed significant differences between the microbial community structures of the ley/arable and grassland field sites. However significant treatment effects were also observed indicating that changes in the bacterial, archaeal, and fungal community structures could still be seen more than 10 years after the final applications of sludge were made. Application of the Zn sludge treatment predominantly affected bacterial communities, with progressively weaker effects observed for archaea and fungi. Conversely, application of the Cu sludge treatment, appeared to cause the greatest changes in fungal and archaeal communities. The extent of the change was also found to be proportional to the total concentrations of Zn and Cu, with significant changes observable at metal concentrations below the current UK statutory limits (Macdonald et al., 2011). Though, Anderson et al. (2008) found no significant treatment effect for Zn, Cu, or Cd, on fungal communities at the Hartwood field site, and suggest that the observed changes were due to the type of sludge applied (digested or undigested) rather than increasing metal contamination. Further investigation by Singh et al. (2014), analysing bacterial 16S rRNA genes and the PLFA profiles of the Scottish field sites has shown significant changes in the bacterial community structure in soils receiving the Zn and Cu sludge treatments at dose-response levels 1 and 4. Significant changes in PLFA profile have also been observed in soils receiving the Zn and Cu sludge treatments at dose-response level 3 at both of these sites, as well as at Woburn and Gleadthorpe (Charlton, Unpublished Data). Despite these changes in community structure, Singh et al. (2014) saw no change in the basal and substrate induced respiration of the contaminated soils, indicating that general metabolic function was unaffected. However, the mineralisation of specific pesticide compounds was impaired, indicating the loss of some specialised functions (Singh et al., 2014). Chaudri et al. (2008) have also reported significant decreases in the legume symbiont *Rhizobium*

*leguminosarum* biovar *trifolii*, and a reduction in N<sub>2</sub>-fixation, in soils receiving the Zn sludge treatment, particularly at the Hartwood field site. Hence the decreases in C<sub>mic</sub> observed during the current investigation do appear to correspond to significant changes in microbial community structure and the disruption of specialised functions within sludge amended soils.

## 5. Conclusions

The results presented here show that significant decreases in soil microbial biomass carbon have occurred in soils where the total concentrations of Zn and Cu fall below the current UK statutory limits. For soils receiving sewage sludge predominantly contaminated with Zn, decreases of approximately 7–11% were observed at concentrations below the UK statutory limit for Zn. In addition, the effect on C<sub>mic</sub> appeared to increase over time, with greater decreases in C<sub>mic</sub> observed over a period of 8 years. This may be due to an interactive effect between Zn and confounding Cu contamination which has augmented the bioavailability of these metals over time. Similar decreases (7–12%) in C<sub>mic</sub> were observed in soils receiving sewage sludge predominantly contaminated with Cu with C<sub>mic</sub> showing signs of recovery after a period of 6 years. In both cases, the observed decreases in C<sub>mic</sub> correspond to significant changes in microbial community structure within the contaminated soils. Application of sewage sludge predominantly contaminated with Cd appeared to have no effect on C<sub>mic</sub> at concentrations below the current UK statutory limit.

## Acknowledgements

This research was funded by the European Union Seventh Framework Programme (FP7-ENV.2010.3.1.1–2 ENV) under grant agreement n° 265269. Alex Charlton also received part funding from the EPSRC Doctoral Training Account. Rothamsted Research receives strategic funding from the UK Biotechnology and Biological Sciences Research Council. Colin D. Campbell is funded by the Scottish Government Rural Affairs and Analytical Services Division (RESAS). We would also like to acknowledge the team from ADAS for their contribution to background data collection. This paper is in memory of the late Professor Brian Chambers of ADAS, without whose dedication, insight, and drive, the UK Long term Sludge Network on which this paper is based would not have been possible.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2016.07.050>.

## References

Abaye, D.A., Lawlor, K., Hirsch, P.R., Brookes, P.C., 2005. Changes in the microbial community of an arable soil caused by long-term metal contamination. *Eur. J. Soil Sci.* 56 (1), 93–102.

Alloway, B.J., 1995. Soil Processes and the behaviour of heavy metals. In: Alloway, B.J. (Ed.), *Heavy Metals in Soils*, second ed. Blackie Academic and Professional, Glasgow, pp. 11–37.

Alloway, B.J., Jackson, A.P., 1991. The behaviour of heavy metals in sewage sludge-amended soils. *Sci. Total Environ.* 100, 151–176.

Anderson, T., Domsch, K.H., 1993. The metabolic quotient for CO<sub>2</sub> ( qCO<sub>2</sub> ) as a specific activity parameter to assess the effects of environmental conditions, such as pH, on the microbial biomass of forest soils. *Soil Biol. Biochem.* 25 (3), 393–395.

Anderson, I.P., Parkin, P.I., Campbell, C.D., 2008. DNA- and RNA-derived assessments of fungal community composition in soil amended with sewage sludge rich in cadmium, copper and zinc. *Soil Biol. Biochem.* 40 (9), 2358–2365.

Anderson, J.A.H., Hooper, M.J., Zak, J.C., Cox, S.B., 2009. Molecular and functional assessment of bacterial community convergence in metal-amended soils. *Microb. Ecol.* 58, 10–22.

Barrow, M.L., Webber, J., 1972. Trace elements in sewage sludges. *J. Sci. Food Agric.* 23 (1), 93–100.

Borenstein, M., 2000. The shift from significance testing to effect size estimation. In: *Comprehensive Clinical Psychology*, vol. 3. Pergamon, Oxford, UK, pp. 313–349.

Borenstein, M., Hedges, L.V., Higgins, J.P.T., Rothstein, H.R., 2009. *Introduction to Meta-analysis*. John Wiley & Sons Ltd, Chichester, UK.

Broos, K., Macdonald, L.M., Warne, M.St.J., Heemsbergen, D.A., Barnes, M.B., Bell, M., McLaughlin, M.J., 2007. Limitations of soil microbial biomass carbon as an indicator of soil pollution in the field. *Soil Biol. Biochem.* 39, 2693–2695.

Brookes, P.C., 1995. The use of microbial parameters in monitoring soil pollution by heavy metals. *Biol. Fertil. Soils* 19 (4), 269–279.

Brookes, P.C., McGrath, S.P., 1984. Effects of metal toxicity on the size of the soil microbial biomass. *J. Soil Sci.* 35 (2), 341–346.

CEC, 1986. Council Directive EU Sewage Sludge Directive 86/278/EEC of 12 June 1986 on the protection of the environment and the soil when sewage sludge is used in agriculture. *Official J. Eur. Comm.* L181.

CEC, 2010. Working Document Sludge and Biowaste 21st September 2010 DG ENV C2/BZ/tb. European Union, Brussels.

Chander, K., Brookes, P.C., 1991. Effects of heavy metals from past applications of sewage sludge on microbial biomass and organic matter accumulation in a sandy loam and silty loam U.K. soil. *Soil Biol. Biochem.* 23 (10), 927–932.

Chander, K., Brookes, P.C., 1993. Residual effects of zinc, copper, and nickel in sewage sludge on microbial biomass in a sandy loam. *Soil Biol. Biochem.* 25 (9), 1231–1239.

Chander, K., Dyckmans, J., Joergensen, R., Meyer, B., Raubach, M., 2001. Different sources of heavy metals and their long-term effects on soil microbial properties. *Biol. Fertil. Soils* 34 (4), 241–247.

Chaudri, A.M., McGrath, S.P., Gibbs, P., Chambers, B., Carlton-Smith, C., Bacon, J., Campbell, C., Aitken, M., 2008. Population size of indigenous *Rhizobium Leguminosarum* Biovar *Trifolii* in long-term field experiments with sewage sludge cake, metal-amended liquid sludge or metal salts: effects of zinc, copper and cadmium. *Soil Biol. Biochem.* 40, 1670–1680.

Dahlin, S., Witter, E., Mårtensson, A., Turner, A., Bååth, E., 1997. Where's the limit? Changes in the microbiological properties of agricultural soils at low levels of metal contamination. *Soil Biol. Biochem.* 29 (9), 1405–1415.

Defra, 2002. Effects of Sewage Sludge Applications to Agricultural Soils on Soil Microbial Activity and the Implications for Agricultural Productivity and Long-term Soil Fertility: Phase II. SP0125. UK Water Industry Research Limited.

Defra, 2007a. Effects of Sewage Sludge Applications to Agricultural Soils on Soil Microbial Activity and the Implications for Agricultural Productivity and Long-term Soil Fertility: Phase III. SP0130. UK Water Industry Research Limited.

Defra, 2007b. Waste Strategy for England 2007. Available from: <http://www.defra.gov.uk/environment/waste/strategy/strategy07/documents/waste07-strategy.pdf>.

Defra, 2008. Long-term Metal Experiments: Statistical Review of Findings to Date and Definition of Future Experimental Programme. SP0142.

Dick, R.P., 1994. Soil enzyme activities as indicators of soil quality. In: Doran, J.W., Coleman, D.C., Bezdjcek, D.F., Stewart, B.A. (Eds.), *Defining Soil Quality for a Sustainable Environment*. Soil Science Society of America and American Society of Agronomy, Madison, WI, pp. 107–124.

DIN, 1997. Extraction von Spurenelementen mit Ammoniumnitratlösung. Deutsche Norm, DIN 19730. Deutsche Institut für Normung, Berlin.

DoE, 1996. Code of Practice for Agricultural Use of Sewage Sludge. Available from: <http://webarchive.nationalarchives.gov.uk/20130402151656/http://archive.defra.gov.uk/environment/quality/water/waterquality/sewage/documents/slugde-cop.pdf> (accessed 06.04.15).

Doran, J.W., Safley, M., 1997. Defining and assessing soil health and sustainable productivity. In: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R. (Eds.), *Biological Indicators of Soil Health*. CAB International, Wallingford, pp. 1–28.

Fließbach, A., Martens, R., Reber, H.H., 1994. Soil microbial biomass and microbial activity in soils treated with heavy metal contaminated sewage sludge. *Soil Biol. Biochem.* 26 (9), 1201–1205.

Hedges, L.V., Gurevitch, J., Curtis, P.S., 1999. The meta-analysis of response ratios in experimental ecology. *Ecology* 80 (4), 1150–1156.

Gendebien, A., Carlton-Smith, C., Izzo, M., Hall, J.E., 1999. UK Sewage Sludge Survey: National Presentation. R & D Technical Report P165. Swindon.

Gendebien, A., Davis, B., Hobson, J., Palfrey, R., Pitchers, R., Rumsby, P., Carlton-Smith, C., Middleton, J., 2010. Environment, Economic, and Social Impacts of the Use of Sewage Sludge on Land: Final Report. Part III: Project Interim Reports. Milieu Ltd., WRc, RPA. [http://ec.europa.eu/environment/archives/waste/slugde/pdf/part\\_iii\\_report.pdf](http://ec.europa.eu/environment/archives/waste/slugde/pdf/part_iii_report.pdf).

Gibbs, P.A., Chambers, B.J., Chaudri, A.M., McGrath, S.P., Carlton-Smith, C.H., Bacon, J.R., Campbell, C.D., Aitken, M.N., 2006. Initial results from a long-term, multi-site field study of the effects on soil fertility and microbial activity of sludge cakes containing heavy metals. *Soil Use Manag.* 22, 11–21.

Giller, K.E., Witter, E., McGrath, S.P., 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. *Soil Biol. Biochem.* 30 (10–11), 1389–1414.

Giller, K.E., Witter, E., McGrath, S.P., 1999. Assessing risks of heavy metal toxicity in agricultural soils: do microbes matter? *Hum. Ecol. Risk Assess.* Int. J. 5 (4), 638–689.

Giller, K.E., Witter, E., McGrath, S.P., 2009. Heavy metals and soil microbes. *Soil Biol. Biochem.* 41 (10), 2031–2037.

Hedges, L.V., Olkin, I., 1980. Vote-counting methods in research synthesis. *Psychol. Bull.* 88 (2), 359–369.

- Hedges, L.V., Pigott, T.D., 2001. The power of statistical tests in meta-analysis. *Psychol. Methods* 6 (4), 203–217.
- Hedges, L.V., Vevea, J.L., 1998. Fixed- and random-effects models in meta-analysis. *Psychol. Methods* 3 (4), 486–504.
- Insam, H., Parkinson, D., 1989. Influence of macroclimate on soil microbial biomass. *Soil Biol. Biochem.* 21 (2), 211–221.
- Jenkinson, D.S., 1977. The soil biomass. *N. Z. Soil News* 25, 213–218.
- Jenkinson, D.S., Ladd, J.N., 1981. Microbial biomass in soil: measurement and turnover. In: A.Paul, E., Ladd, J.N. (Eds.), *Soil Biochemistry*, vol. 5. Marcel Dekker, New York, pp. 415–471.
- Jenkinson, D.S., Brookes, P.C., Powlson, D.S., 2004. Measuring soil microbial biomass. *Soil Biol. Biochem.* 36, 5–7.
- Khan, M., Scullion, J., 1999. Microbial activity in grassland soil amended with sewage sludge containing varying rates and combinations of Cu, Ni and Zn. *Biol. Fertil. Soils* 30, 202–209.
- Kim, B., McBride, M.B., 2009. Phytotoxic effects of Cu and Zn on soybean grown in field-aged soils: their additive and interactive actions. *J. Environ. Qual.* 38, 2253–2259.
- Lewis, S., Clarke, M., 2001. Forest plots: trying to see the wood and the trees. *Br. Med. J.* 322, 1479–1480.
- Macdonald, C.A., Clark, I.M., Zhao, F.-J., Hirsch, P.R., Singh, B.K., McGrath, S.P., 2011. Long-term impacts of zinc and copper enriched sewage sludge additions on bacterial, archaeal, and fungal communities in arable and grassland soils. *Soil Biol. Biochem.* 43, 932–941.
- MAFF, 1986. *Analysis of Agricultural Materials*. Reference Book 427, third ed. HMSO, London.
- MAFF/DoE, 1993a. Review of the Rules for Sewage Sludge Application to Agricultural Land: Food Safety and Relevant Animal Health Aspects of Potentially Toxic Elements: Report of the Steering Group on Chemical Aspects of Food Surveillance. PB 1562. MAFF Publications, London.
- MAFF/DoE, 1993b. Review of the Rules for Sewage Sludge Application to Agricultural Land: Soil Fertility Aspects of Potentially Toxic Elements. Independent Scientific Committee on Soil Fertility Aspects of Sewage Sludge Use in Agriculture. PB 1561. MAFF Publications, London.
- Martens, R., 1995. Current methods for measuring microbial biomass C in soil: potentials and limitations. *Biol. Fertil. Soils* 19 (2–3), 87–99.
- McBride, M.B., 1995. Toxic metal accumulation from agricultural use of sludge: are USEPA regulations protective? *J. Environ. Qual.* 24, 5–18.
- McBride, M.B., 2003. Toxic metals in sewage sludge-amended soils: has promotion of beneficial use discounted the risks? *Adv. Environ. Res.* 8 (1), 5–19.
- McLaren, R.G., Crawford, D.V., 1973. Studies on soil copper II. The specific adsorption of Cu by soils. *J. Soil Sci.* 24 (4), 443–452.
- McGrath, S.P., 1984. Metal concentrations in sludges and soil from a long-term field trial. *J. Agric. Sci.* 103 (1), 25–35.
- McGrath, S.P., 1987. Long-term studies of metal transfers following application of sewage sludge. In: Coughtry, P.J., Martin, M.H., Unsworth, M.H. (Eds.), *Pollutant Transport and Fate in Ecosystems*, Special Publication Number 6 of the British Ecological Society. Blackwell Scientific, Oxford, pp. 25–35.
- McGrath, S.P., Chang, A.C., Page, A.L., Witter, E., 1994. Land application of sewage sludge: scientific perspectives of heavy metal loading limits in Europe and the United States. *Environ. Rev.* 2 (1), 108–118.
- McGrath, S.P., Chaudri, A.M., Giller, K.E., 1995. Long-term Effects of metals in sewage sludge on soils, microorganisms, and plants. *J. Ind. Microbiol.* 14 (2), 94–104.
- McGrath, S.P., Cunliffe, C.H., 1985. A simplified method for the extraction of the metals Fe, Zn, Ni, Cd, Pb, Cr, Mo, and Mn from soils and sewage sludges. *J. Sci. Food Agric.* 36, 794–798.
- Moja, L., Moschetti, I., Liberati, A., Gensini, G.F., Gusinu, R., 2007. Understanding systematic reviews: the meta-analysis graph (also called ‘forest plot’). *Intern. Emerg. Med.* 2 (2), 140–142.
- Powlson, D.S., Brookes, P.C., 1987. Measurement of soil microbial biomass provides an early indication of changes in total soil organic matter due to straw incorporation. *Soil Biol. Biochem.* 19 (2), 159–164.
- Pueyo, M., López-Sánchez, J.F., Rauret, G., 2004. Assessment of CaCl<sub>2</sub>, NaNO<sub>3</sub> and NH<sub>4</sub>NO<sub>3</sub> extraction procedures for the study of Cd, Cu, Pb, and Zn extractability in contaminated soils. *Anal. Chim. Acta.* 504, 214–226.
- Ritz, K., Black, H.I.J., Campbell, C.D., Harris, J.A., Wood, C., 2009. Selecting biological indicators for monitoring soils: a framework for balancing scientific and technical opinion to assist policy development. *Ecol. Indic.* 9, 1212–1221.
- Schlöter, M., Dilly, O., Munch, J.C., 2003. Indicators for evaluating soil quality. *Agric. Ecosyst. Environ.* 98 (1–3), 255–262.
- Schutter, M.E., Sandeno, J.M., Dick, R.P., 2001. Seasonal, soil type, and alternative management influences on microbial communities of vegetable cropping systems. *Biol. Fertil. Soils* 34, 397–410.
- SI, 1989. *The Sludge (Use in Agriculture) Regulations 1989*, No 1263. Available from: <http://www.legislation.gov.uk/ukxi/1989/1263/made>.
- Singh, B.K., Quince, C., Macdonald, C.A., Kachane, A., Thomas, N., Al-Soud, W.A., Sørensen, S.J., He, Z., White, D., Sinclair, A., Crooks, B., Zhou, J., Campbell, C.D., 2014. Loss of microbial diversity in soils is coincident with reductions in specialized functions. *Environ. Microbiol.* 16 (8), 2408–2420.
- Smith, S.R., 1996. *Agricultural Recycling of Sewage Sludge and the Environment*. CAB International, Wallingford.
- Stockdale, E.A., Brookes, P.C., 2006. Detection and quantification of the soil microbial biomass - impacts on the management of agricultural soils. *J. Agric. Sci.* 144, 285–302.
- Thornton, I., Butler, D., Docx, P., Hession, M., Makropoulos, C., McMullen, M., Nieuwenhuijsen, M., Pitman, A., Rauti, R., Sawyer, R., Smith, S., White, D., 2001. *Pollutants in Urban Waste Water and Sewage Sludge*. Final Report to Directorate-General Environment. Office for Official Publications of the European Communities, Luxembourg.
- Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. *Soil Biol. Biochem.* 19 (6), 703–707.
- Water UK, 2010. *Recycling of Biosolids to Agricultural Land*. Water UK, London, SW1H 9BT.
- Wu, Y., Kemmitt, S., White, R.P., Xu, J., Brookes, P.C., 2012. Carbon dynamics in a 60 year fallowed loamy-sand soil compared to that in a 60 year permanent arable or permanent grassland UK soil. *Plant Soil* 352, 51–63.
- Zelles, L., 1999. Fatty acid patterns of phospho-lipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. *Biol. Fertil. Soils* 29, 111–129.